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UNIVERSITY OF ALBERTA

MECHANISMS OF CEREBRAL VASOSPASM FOLLOWING SUBARACHNOID HEMORRHAGE IN MONKEYS

BY

Robert Loch Macdonald

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

FALL 1991

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Kenichi Saito, M.D.

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MJP/am

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R. L. Macdonald /am

R. Loch Macdonald, M.D.

RLM/am

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Thank you in advance for your consideration of this matter.

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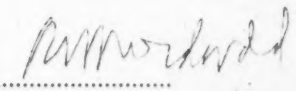

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Date: May 24, 1991

This work is dedicated to my wife, Sheilah,
my son, Iain Alexander, and
to my parents Lea and Neil

ABSTRACT

The role of hemoglobin in generating cerebral vasospasm was evaluated in a study where forty cynomolgus monkeys were divided into 5 groups and given intrathecal injections, twice a day for 6 days, of one of the following solutions: 1. oxyhemoglobin, 2. methemoglobin, 3. bilirubin, 4. mock cerebrospinal fluid (CSF), and 5. supernatant fluid from an incubated autologous blood-mock CSF mixture. Significant vasospasm of middle cerebral arteries, as assessed by comparison of day 7 and day 0 angiograms, developed in animals injected with oxyhemoglobin and supernatant fluid ($p < 0.05$, paired t-test). These data suggest oxyhemoglobin causes cerebral vasospasm following subarachnoid hemorrhage. Cerebrospinal fluid levels of malondialdehyde, a product of lipid peroxidation, were elevated after injection of oxyhemoglobin and supernatant fluid, suggesting that lipid peroxidation accompanies vasospasm induced by oxyhemoglobin.

To study if oxyhemoglobin-induced vasospasm is mediated by free radicals, 17 monkeys were randomized to 4 groups to receive subarachnoid placement of either 1. agarose, 2. agarose plus oxyhemoglobin, 3. agarose plus oxyhemoglobin plus treatment with intrathecal catalase and human recombinant superoxide dismutase, or 4. agarose plus oxyhemoglobin plus treatment with placebo. Comparison of baseline angiography with angiography performed 7 days later showed oxyhemoglobin alone significantly reduced middle cerebral artery diameter ($p < 0.005$, paired t-test). Treatment with either superoxide dismutase plus catalase or with placebo attenuated arterial narrowing, compared with effects of oxyhemoglobin alone, although significant middle cerebral artery vasospasm ($p < 0.05$, paired t-test) persisted in each group and vasospasm was not significantly different between groups at day 7 (analysis of variance). These findings suggest either that free radical-mediated reactions are not critical to development of oxyhemoglobin-induced arterial narrowing or the particular dosing regimen employed was not efficacious.

To see if vasospasm due to whole blood or to oxyhemoglobin was associated with increased arterial wall area, computerized image-processing was used to measure lumen area, total vessel wall area, and area of tunica media plus tunica intima of fixed cross-sections of monkey middle cerebral arteries exposed *in vivo* for 6 days to whole blood, oxyhemoglobin, methemoglobin, bilirubin, mock CSF, or supernatant fluid. Significant increases in total wall area of right compared to left middle cerebral arteries developed in arteries exposed to oxyhemoglobin, bilirubin, and mock CSF ($p < 0.05$, paired t-test). There were no significant changes in area of tunica media plus tunica intima. Increased wall area was due to deposition of inflammatory debris in the tunica adventitia. This latter finding was nonspecific, occurring after injection of agents not associated with development of vasospasm (for example, mock CSF). The results suggest that proliferative changes in vasospastic arteries do not contribute primarily to lumen narrowing.

Arterial smooth muscle cells were studied by scanning electron microscopy after HCl hydrolysis of arteries constricted *in vitro* with prostaglandin $F_{2\alpha}$ or exposed *in vivo* for one week to whole blood or oxyhemoglobin. Smooth muscle cells contracted *in vitro* exhibited extensive cell membrane pleating and appeared similar to muscle cells from arteries made vasospastic with oxyhemoglobin or whole blood. The similar morphology of vasospastic and vasoconstricted smooth muscle suggests smooth muscle is contracted during vasospasm.

Nine monkeys had bilateral subarachnoid clots placed and equal numbers were killed 7, 14, and 28 days later. Both middle cerebral arteries and control basilar arteries were studied using immunohistochemistry with primary antibodies to actin, myosin, vimentin, desmin, fibronectin, fibrinogen, factor 8-related antigen, laminin, and collagens (types I, III, IV, and V). Study of slides at microscopy and blinded review of photographs of arteries by 5 independent observers showed no qualitative changes in intensity or pattern of staining for any antigens except for increased fibrinogen immunoreactivity on day 7, increased fibronectin immunoreactivity on day 14, and intimal thickening at 28 days after SAH which contained cells with immunoreactivity to α-actin, myosin, desmin, and vimentin and extracellular matrix

with immunoreactivity to fibronectin, laminin, and each type of collagen. Total collagen content of arteries, as measured by hydroxyproline content, did not change at any time after subarachnoid hemorrhage. These findings suggest that vasospasm is primarily smooth muscle contraction and that pathologic changes, such as intimal proliferation and fibronectin deposition are a secondary response of the arterial wall to injury which occurs during vasospasm.

KEY WORDS

Bilirubin; Free Radicals; Immunohistochemistry; Methemoglobin; Oxyhemoglobin; Subarachnoid Hemorrhage; Vasospasm

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CHAPTER ONE [a]
HEMOGLOBIN AND CEREBRAL VASOSPASM

Rupture of an intracranial saccular aneurysm is the fourth most common cause of stroke.[285] Autopsy studies show approximately 5% of adults harbour such aneurysms and each year 10 of every 100,000 people will suffer from rupture of a saccular aneurysm. Hemorrhage from these aneurysms deposits blood in the subarachnoid space (subarachnoid hemorrhage, SAH) through which the major cerebral arteries course prior to entering the brain. If patients survive the initial bleeding, they are prone to develop cerebral vasospasm (VSP) which is a delayed onset and sustained narrowing of cerebral arteries as they pass through the blood clot to supply the brain. Vasospasm may result in brain ischemia and death.[267,269,285]

OCCURRENCE AND CLINICAL FEATURES

Vasospasm may be detected on 30% to 70% of angiograms performed 4 to 12 days after aneurysmal SAH. Weir et al, in a review of 627 angiograms performed on 293 patients with SAH, found VSP had its onset 3 days after SAH, was maximal on days 6 to 8 and resolved by day 12.[268] Delayed onset of VSP is characteristic and, although in animal models of SAH vasoconstriction occurs immediately upon induction of hemorrhage, acute vessel narrowing is probably unimportant in man.[269,273]

Development of VSP correlates best with volume and location of SAH. Fisher et al reported that thick collections of subarachnoid blood on a computed tomography (CT) scan performed within 5 days of SAH were associated with an incidence of vasospasm of 96%.[66] If no blood was seen on CT, fewer than 5% of patients developed VSP. Clinical condition of the patient worsens as volume of subarachnoid blood increases and it follows that patients at greatest risk of developing VSP are those who are most damaged by the initial SAH.

Delayed ischemic neurologic deficits develop in 20% to 30% of patients with VSP.[65,91,113,114,149,269,284] Whether brain infarction occurs depends not only on severity and distribution of VSP but also on other factors which control cerebral perfusion such as blood pressure, blood volume, blood viscosity, intracranial pressure, collateral cerebral circulation, and pre-existing cerebrovascular occlusive disease.[284]

Patients with VSP commonly exhibit low-grade fever, increasing headache and neck stiffness, and deterioration in level of consciousness.[284] Peripheral blood leucocytosis and electrocardiographic abnormalities may also occur.[65,113,149,270] Focal neurologic deficits often develop in the territory of vasospastic vessels. Diffuse VSP of all arteries of the circle of Willis may cause death due to global cerebral ischemia.[113,149,284]

DIAGNOSIS

Patients with SAH may deteriorate neurologically from aneurysm rebleeding into the subarachnoid or intracerebral compartments, obstructive hydrocephalus, seizures, cerebral edema, postoperative clots, electrolyte imbalance (most commonly hyponatremia), sepsis, or hypoxemia.[113,149,284] In deteriorating patients, investigations should be performed to detect these as well as any other medical conditions which could impair supply of oxygen and glucose to the brain.

In addition, CT scanning is indicated emergently as it will readily detect new collections of blood as well as focal hypodensity suggestive of cerebral edema or infarction. Thick cisternal blood collections on CT also predict patients at risk of

---

[a] A version of this chapter has been accepted for publication. Macdonald RL, Weir BKA: A review of hemoglobin and the pathogenesis of cerebral vasospasm. Stroke 1991 (in press).

developing VSP.[66] Magnetic resonance imaging is difficult to perform in critically-ill patients and contraindicated in those with ferromagnetic aneurysm clips although it is very sensitive in detecting increased brain water which accompanies cerebral infarction.[191] Careful selection of repetition and echo times allow magnetic resonance imaging to be as sensitive as CT in detecting SAH, especially in the posterior fossa, and possibly more sensitive than CT in detecting the source of bleeding.[103,191]

Definitive diagnosis of VSP has traditionally required cerebral angiography. Spastic vessels have reduced diameter on angiography, usually with smooth tapering of the lumen, although abrupt focal narrowing may be observed.[149] Single or multiple cerebral arteries may be involved over short or long segments. Non-invasive techniques, chiefly transcranial Doppler ultrasound, are currently being used to diagnose VSP.[1,81,98,210] Transcranial Doppler measures flow velocity and since velocity is approximately proportional to the square of vessel diameter, this technique is very sensitive in detection of arterial narrowing. Incidence of vessel narrowing after aneurysmal SAH as detected by transcranial Doppler has been as high as 93% [81] and flow velocity elevation has been found as early as 2 days after SAH.[210] Furthermore, transcranial Doppler is non-invasive, devoid of complications, and easy to perform repeatedly.

TREATMENT

In 1986, Wilkins reviewed treatments and preventive protocols for VSP and concluded that, although some gains had been made, the problem was still largely unsolved.[283] The most promising avenues were considered to be early operation with aneurysm obliteration and removal of as much subarachnoid clot as possible, measures to maximize cerebral perfusion, and administration of Ca^{2+}-channel antagonists. Treatments for VSP may be broadly divided into therapy directed at preventing or reversing arterial narrowing and therapy directed at preventing or reversing cerebral ischemia.[64]

Treatment of Arterial Narrowing

Removal of Subarachnoid Clot: Despite prevailing opinion that early surgical treatment of ruptured cerebral aneurysm was technically more difficult and sometimes more dangerous than delaying operation, Suzuki et al [228] and others [148] advocated the former approach since rebleeding could be prevented and intracranial pressure could be safely controlled by ventricular drainage. In addition, they felt removal of subarachnoid clot lessened the risk of VSP. Experiments in a monkey model of SAH showed that evacuation of subarachnoid clot within 48 hours of placement prevented VSP.[88,164] In these studies, hematomas were removed by surgery, a procedure which could not be easily performed in humans. Subsequent work using the monkey model of SAH showed it was possible to pharmacologically lyse the subarachnoid clot with intrathecal recombinant tissue plasminogen activator, thereby preventing VSP.[60,61] Use of recombinant tissue plasminogen activator to prevent VSP in humans has been reported although randomized, controlled, blinded studies have not yet been performed.[63,130,237]

Pharmacologic Treatment of Arterial Narrowing: Although smooth muscle contraction has been thought to underlie VSP, failure of vasodilator drugs to prevent or reverse VSP in humans suggests other pathophysiologic processes are important.[114] Since cerebral arteries constrict upon influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels, treatment of VSP with Ca^{2+}-channel antagonists, principally nimodipine, has been commonly reported. Although nimodipine does not affect angiographically-visible VSP in monkeys [55,163] or humans,[8,188] it is accepted that when given prophylactically it does improve outcome following SAH. An alternate mechanism of action has been suggested to be prevention of ischemic brain damage by inhibiting Ca^{2+} influx into ischemic neurons, although it may also benefit patients by dilating smaller resistance arteries and collateral pathways not visible on angiograms.[211]

Failure of nimodipine to reverse VSP is consistent with experimental studies of spastic arteries which show these arteries are stiffer and less compliant than normal arteries.[121] Opinion is divided, however, and there are reports of pharmacologic reversal of VSP both in animals [156] and humans.[64] Furthermore, results of a controlled, clinical trial of nicardipine in aneurysmal SAH showed that continuous infusion of high doses of nicardipine significantly diminished angiographic VSP.[83,115] This is the first drug shown conclusively to affect VSP in humans.[67] Despite this, outcome was not significantly different between groups and the investigators suggested this was because hypertensive, hypervolemic, hemodilution therapy, which was used more often in the placebo group, was just as effective in preventing cerebral ischemia due to VSP.

Based on the hypothesis that VSP is an inflammatory vasculopathy mediated by vasoconstricting eicosanoids, Chyatte et al treated VSP in dogs with both nonsteroidal and high-dose steroidal anti-inflammatory drugs.[34] Promising results were obtained and they went on to use these agents in humans in an open cohort study which again resulted in improved outcome in treated patients.[35] Results of treatment of VSP with the immunosuppressive drug cyclosporine have produced conflicting results.[89,187]

Another drug with a demonstrated effect on VSP in an experimental model is U74006F, a 21-aminosteroid compound which inhibits iron-dependent lipid peroxidation.[27] Intravenous U74006F significantly decreased VSP in a monkey model of SAH.[222] Although this drug may have other actions, results suggest a role for free radical mechanisms in VSP and warrant more experimentation with free radical scavengers, anti-oxidants, iron chelators,[259] and agents which bind hemoglobin such as haptoglobin.

Percutaneous Transluminal Angioplasty: With the advent of sophisticated endovascular techniques, it is possible to guide small balloon catheters into major cerebral arteries. Dilating vasospastic arteries by inflation of balloons within them, termed balloon angioplasty, has been reported as a treatment for medically intractable symptomatic VSP.[15,92,159,296] This procedure requires skill and experience, and is not without complications, including potential rupture of angioplastied arteries and of the previously ruptured aneurysm itself, if it has not been obliterated.

Treatment of Cerebral Ischemia

Attempts to Increase Cerebral Perfusion: Improved blood supply to ischemic brain distal to a vasospastic artery of fixed diameter may be accomplished by increasing pressure at the proximal end of the vessel or by decreasing blood viscosity.[112,149] Cerebral perfusion pressure can be increased by intravascular volume expansion and by elevating cardiac output and blood pressure with inotropes, with careful attention paid to intracranial pressure. Such hypertensive, hypervolemic, hemodilutional therapy is credited with decreasing the incidence of cerebral ischemia in patients with VSP.[112,283] Although volume expansion usually ensures low blood viscosity, the primary effect of volume expansion has usually been believed to be via elevation of cardiac output.[24] Wood et al, however, studied focal cerebral ischemia following cerebral arterial occlusions in dogs.[287] Although volume expansion with whole blood elevated cardiac output, cerebral blood flow and infarction remained unchanged. They concluded that reduction of blood viscosity, which did not occur after infusion of whole blood, was important in the mechanism of action of volume expansion. Further evidence was supplied by Bouma et al who found no correlation between cardiac output and cerebral blood flow in head-injured patients whose blood pressure was manipulated pharmacologically.[24] Their data suggested the effect of intravascular volume expansion was by decreased blood viscosity and not by cardiac output augmentation.

Surgical methods to improve blood flow to ischemic cerebral tissue include extracranial-intracranial bypass grafting and this has been employed for intractable symptomatic VSP.[196]

Brain Protection: It is possible to make brain tissue more resistant to ischemia or hypoxia using drugs such as barbiturates. Trials of barbiturates in patients with VSP

after SAH have had poor results although these drugs were usually used as a last resort in critically-ill patients.[110,211,288] Nimodipine improves outcome following SAH although it does not decrease severity of VSP; one mechanism of action to explain decreases in ischemic deficits after treatment with nimodipine has been brain protection.[211] Both nimodipine and barbiturates affect cerebral blood flow, making it difficult to define the exact mechanism of benefit, if any, from these agents.[211]

PATHOGENESIS

It is established that subarachnoid blood causes VSP, independent of any direct vascular injury incurred at the time of SAH. Mechanisms of arterial narrowing, however, remain ill-defined. Whether lumen narrowing is due to contraction of muscle cells,[62] to increase in arterial wall tissue,[37,111] the excess tissue encroaching on the lumen, or to some combination of these two mechanisms is unclear. Other pathologic processes such as arterial wall fibrosis and inflammation have been suggested to contribute to prolongation of arterial narrowing.[18] Furthermore, identity of the substance or substances released from clot which interact in some way with the vessel wall to produce lumen narrowing are not established with certainty. Biochemical and molecular basis of processes transpiring in the arterial wall during onset, course, and resolution of VSP remain almost completely undefined.[127,185,231,260]

Attempts to Isolate the Spasmogen

Incubation of blood *in vitro* results in slow hemolysis of erythrocytes with release of oxyhemoglobin (OxyHb) into the supernatant fluid starting after 2 days.[11,16,45,146,176,179,199,218,219] With time, OxyHb is oxidized to methemoglobin (MetHb)[11,16,176,218,219] but further breakdown of heme to bilirubin probably occurs only *in vivo*.[16,195] Only Duff et al reported increasing amounts of bilirubin were produced by clotted blood incubated *in vitro*.[46] Exactly how heme groups of hemoglobin (Hb) are broken down into bilirubin are not known but they probably require enzymes present only in living cells.[285] After SAH, red blood cells (RBC) remain fixed in the subarachnoid space for days and they disappear by hemolyzing, similar to the process *in vitro*.[16,61]

In 1944, Zucker recognized that RBC cytosol was vasoactive although platelets and serum were much more potent vasoconstrictors.[297] Further experiments have documented that erythrocytes contain a vasoactive substance.[32,87,99,109,143,146,172,176,179,180,199,218-220,281]

Jackson injected fresh and aged blood components into the cisterna magna of dogs.[101] Animals were observed for signs of meningeal irritation and cerebrospinal fluid (CSF) was analyzed for inflammatory response. Autologous serum or plasma incubated at body temperature for 4 to 9 days and autologous, fresh, whole blood did not cause meningeal irritation and resulted in only a mild cell response in CSF. Whole blood or packed RBCs incubated *in vitro* produced marked meningeal irritation, fever, and a marked cell response in CSF, effects being greatest after incubation of blood or RBCs at body temperature for 3 to 12 days. Intracisternal injection of OxyHb resulted in severe malaise and fever. Jackson concluded the agent in blood causing the greatest meningeal response was present in the heme component of blood. Angiography was not done. Humans with clinically-significant VSP after SAH have higher temperatures and white blood cell counts on average than patients without VSP.[270] Simmonds also found meningeal reactions to cisternal injections in rabbits were more severe with blood than with plasma.[214]

Kapp et al applied fresh blood products to exposed cat basilar artery (BA).[109] Lysed platelets were most active followed by lysed leucocytes, lysed erythrocytes and intact leucocytes. Intact RBCs were least vasoactive. Wilkins and colleagues found that plasma discolored by hemolysis caused more contraction of rabbit aorta *in vitro* than did clear plasma.[281] They also demonstrated in dogs that single intracisternal injections of supernatant fluid from incubated blood caused BA spasm which sometimes lasted for

days.[282] Fresh serum was much less effective. They speculated about the role of vasoconstrictor substances released by hemolysis in generating VSP. Starling et al compared vasocontractile activities of lysed and intact erythrocytes on human BA *in vitro*.[220] Lysis greatly accentuated RBC activity.

Many investigators have assayed vasoactivity of incubated and aged mixtures of whole blood, blood components, and CSF on dog and cat BA *in vitro*.[32,53,87,146,176,179,180,218,219] Results are remarkably concordant between experiments and may be summarized as follows. Fresh serum, platelet-rich plasma, and lysed RBCs have significant vasoactivity whereas fresh, intact erythrocytes are inert. With incubation, serum and platelet-rich plasma become inactive whereas contractile activity of intact or lysed erythrocytes persists. Similar experiments were performed *in vivo* by McIlhany and colleagues who injected vasoactive fractions of hemolyzed RBCs, as collected on Sephadex G-75 gel filtration chromatography, into the cisterna magna of dogs.[143] Vasoconstriction of BA was observed for 2 days. Peterson et al observed reactions 72 hours after subarachnoid injections of blood components into dogs.[186] Erythrocyte lysis was found to be necessary for development of VSP. In summary, during VSP in man, vasoactivity of blood incubated *in vitro*, when tested on BA *in vitro*, resides in erythrocytes. Hemogenic meningitis, clinical signs of which often develop in association with VSP, is most likely due to erythrocyte lysis.

The role of erythrocytes in generating VSP has been evaluated further by long-term *in vivo* studies. Duff and coworkers injected whole blood, serum, or erythrocyte-free blood into the prepontine cistern of cats.[47] Ultrastructural analysis of BA was performed after 7 and 10 days. Whole blood caused luminal narrowing with smooth muscle cell damage and disintegration of adventitia. Arteries of animals injected with serum or RBC-free blood were normal. Mayberg et al developed a piglet model whereby blood components could be selectively applied to middle cerebral arteries (MCA).[142] After 10 days, VSP was assessed by morphometric analysis of MCAs. Whole blood caused significant reduction in lumen diameter. Selective application of washed erythrocytes, RBC cytosol and pure porcine Hb also caused significant VSP whereas leukocytes and platelet-rich plasma did not. In dogs, Nozaki and colleagues reported that VSP of BA developed only after injection of blood components containing RBCs.[166]

The vasoactive substance released by erythrocytes has been isolated by many groups. As early as 1976, Miyaoka et al used ion-exchange resin chromatography and disc electrophoresis to demonstrate that vasoactivity of lysed erythrocytes was due to a peptide equal to or closely allied to OxyHb.[146] Sonobe and Suzuki found the spasmogen isolated from supernatant fluid of incubated blood-CSF mixtures to be OxyHb as assessed by gel chromatography, disc electrophoresis, and spectrophotometry.[218,219] Other attempts at isolation of vasoactive substances from incubated blood or from lysed erythrocytes consistently produced OxyHb or a chemical with similar properties [87,172,176,180] although the molecular weight of the vasoactive compound isolated by Cheung et al [32] (40,000 to 45,000 daltons) is low for Hb which has a molecular weight of 64,500. Lower molecular weight fractions of incubated hemolysates which might contain other potentially vasoactive substances such as lipid peroxides, bilirubin, and endothelin, are not vasoactive, although some of these agents would not be synthesized by blood incubated *in vitro*.[195,286,291] Vasoactivity of blood incubated *in vitro*, however, also correlates with its content of thiobarbituric-acid reactive substances (and with concentration of OxyHb).[11,199,200] Substances that react with thiobarbituric acid include lipid peroxides. Strong spectrophotometric absorption by OxyHb interferes significantly with the thiobarbituric acid test, making interpretation of these results difficult.

These investigations show erythrocytes are essential for development of VSP. Results of studies isolating the spasmogen in erythrocytes point to OxyHb as a prime candidate to cause VSP.

Studies of Cerebrospinal Fluid after SAH

Delayed onset of VSP after SAH could be explained by the spasmogen exhibiting a similar time course of appearance in CSF near arteries in spasm. An alternative hypothesis explains delay in onset by suggesting arterial narrowing is due to another mechanism, such as fibrosis and inflammatory-cell infiltration of the arterial wall [18,35,62,114,141,217,289] or to intimal proliferation,[37,111,184,216] processes which are set in motion by events occurring immediately after SAH and which are related more to the acute effects of SAH than to happenings in CSF at the actual time of VSP. Theories supposing VSP involves these processes are reviewed below.

Pathology of the subarachnoid space following human SAH was studied by Bagley,[14] Hammes,[86] and Alpers and Forster.[9] Their results were similar. Within 24 hours of SAH there was intense polymorphonuclear cell infiltration of the meninges. Phagocytosis and breakdown of RBCs was observed by 16 to 32 hours. Erythrocyte breakdown peaked around day 7 but continued for days with clumps of intact erythrocytes still enmeshed in the arachnoid 35 days after SAH. At 7 days, the inflammatory reaction was subsiding and was composed equally of lymphocytes and phagocytes. After 10 days, fibrosis of the subarachnoid space began. Therefore, during VSP, the most prominent process occurring in the subarachnoid space is RBC hemolysis. Clearance of blood injected into the subarachnoid space of animals is rapid and most erythrocytes are removed by methods other than hemolysis.[2,48,214,215] This is probably why single liquid blood injections in experimental animals fail to mimic human VSP.[269]

Hemolytic breakdown of erythrocytes observed microscopically may also be observed by spectrophotometric examination for pigments released into CSF. Barrows and colleagues used absorption spectrophotometry to examine CSF after SAH.[16] Oxyhemoglobin appeared 2 hours after SAH. By 4 days post-ictus, bilirubin was present and over the following week, OxyHb decreased and bilirubin increased. Without further bleeding, both pigments disappeared after 2 to 3 weeks. Bilirubin is present in normal serum and could be observed in CSF immediately after massive SAH. In cases of SAH, MetHb was not detected.

Kronholm and Lintrup detected MetHb in CSF after "cerebral hemorrhage", stating that Barrows et al did not detect it because they used spectral characteristics in the 500 to 650 nm region where absorption of MetHb is only one fortieth of the maximum at it's Soret band at 406 nm.[128,129]

Van Der Meulen prepared standard solutions containing various combinations of OxyHb, protein, and bilirubin.[254] Previous spectrophotometric investigations of CSF had not accounted for non-specific absorption of protein.[16,128,129] He found that protein concentrations above 100 mg% increased apparent CSF OxyHb concentrations and decreased apparent CSF bilirubin concentrations. Application of these observations showed OxyHb to be present for longer periods after SAH than had previously been thought. Results of these and other studies [251,256,262] show OxyHb, MetHb, and bilirubin are present in CSF during VSP.

Few other substances appear in CSF with a time course similar to that of VSP after SAH. Potentially spasmogenic small molecules and proteins, such as noradrenaline, serotonin, and plasma proteins are cleared rapidly from CSF and are probably unimportant in VSP.[30,212,258,277,278,280] Since VSP correlates best with volume of SAH, elevations of CSF serotonin, for example, might correlate well with VSP but have no causal relation to development of VSP. Fibrin degradation products, which may be vasoactive [134] are elevated after SAH [99] but subarachnoid clot lysis with plasminogen activator, which releases large amounts of degradation products, prevents VSP.[60,61] Vasopressin levels are elevated but are felt to contribute to genesis of cerebral edema and not VSP.[278,280]

Other potentially important spasmogens, based on their appearance in CSF after SAH, are lipid peroxides, endothelin, and some of the eicosanoids. Numerous investigations have demonstrated concentrations of lipid peroxides in lumbar or cisternal CSF are significantly higher in patients with VSP after SAH than in SAH patients

without VSP.[11,199,200,205,266] Lipid peroxides generally display a time course of appearance in CSF which is similar to that of VSP.[11,200,266]

Products of the cycloxygenase arm of arachidonic acid metabolism have been studied in CSF after SAH. Elevated levels of vasoconstrictor prostaglandins E_2 (PGE_2) and $PGF_{2\alpha}$ were found in CSF after SAH in humans [30,82,194] and dogs.[264,265] Thromboxane B_2 (TXB_2) and 6-oxo $PGF_{1\alpha}$ were also slightly elevated.[207,264] Another study found CSF PGD_2 was elevated and CSF 6-keto-$PGF_{1\alpha}$ was decreased in one patient with VSP while these substances remained unchanged in one case without VSP after SAH.[193] Paoletti et al investigated lipoxygenase metabolites in CSF after SAH.[181] Lumbar and cisternal CSF leukotriene C_4 (LTC_4) levels were found in significantly greater concentration in patients with VSP as compared to those without. Leukotriene synthesis was activated in BA following SAH in dogs.[213,292]

Investigation of contractile activity of CSF from patients with ruptured aneurysms was first performed by Buckell in 1964.[28] Some CSF activity could be accounted for by serotonin but in half the cases, other substances were present which caused contraction. Contractile activity of CSF after SAH has since been frequently studied.[6,7,20-22,25,26,42,97,117,177,234] Allen et al found xanthochromic CSF caused dose-dependent contraction of dog and human BA *in vitro*.[7,8] Treatment of one dog BA with phenoxybenzamine prevented contraction, leading them to conclude that the agent in xanthochromic CSF responsible for smooth muscle contraction, and presumably VSP, was serotonin. Tagari and colleagues found ketanserin could partially antagonize contractile effects of xanthochromic CSF on human cerebral arteries and on rat stomach fundi.[234] Most investigators, however, report that drugs which block serotonin, histamine, acetylcholine, norepinephrine, epinephrine, propranolol, prostaglandins, and angiotensin II do not prevent hemorrhagic CSF from contracting cerebral arteries *in vitro*.[21,22,177,204] Calcium-channel antagonists usually at least partially block such contractions.[25,177] This suggests the spasmogen in xanthochromic CSF contracts smooth muscle by a mechanism independent of receptors for these classical transmitters but dependent on influx of Ca^{2+} through voltage-gated Ca^{2+} channels. Evidence reviewed below shows OxyHb-induced contractions have similar pharmacologic properties to contractions caused by xanthochromic CSF.

Sasaki et al further delineated the nature of CSF contractile activity by showing disulphide-bond reducing agents blocked contractions induced by xanthochromic CSF on dog BA.[204] Disulphide-bond reducers are known to block smooth muscle contraction due to prostaglandins, Hb, and lipid peroxides. Attempts to isolate spasmogens from hemorrhagic CSF using chromatography and gel electrophoresis have rarely been made. Yamamoto and coworkers assayed CSF activity by measuring rates of contraction induced in fibroblasts cultured on collagen latticies.[289] The spasmogen was not destroyed by heating and had a molecular weight less than 8000. Papadopoulos et al [182] noted that vasoconstriction due to endothelin, a potent and low molecular weight vasoconstrictor peptide synthesized by endothelial cells,[291] is blocked by disulphide-bond reducing agents but not by antagonists of cell-surface receptors. Endothelin levels are elevated following SAH [139] and increased levels are associated with VSP.[206] Oxyhemoglobin augments release of endothelin from cultured vascular endothelial cells.[135] Because endothelin is a peptide, its production is regulated by control of transcription of its gene and it is possible that after SAH, expression of endothelin genes could be up-regulated. Changes in gene expression can be prolonged.

If vasoactivity in xanthochromic CSF is due to OxyHb, then CSF OxyHb concentration and severity of VSP should correlate. Ohta and coworkers determined total Hb concentration in lumbar CSF of 78 SAH patients.[169] They could not correlate lumbar CSF Hb concentration with VSP. Lumbar CSF Hb concentrations did not exceed 5 g/dL. Analysis of 16 clots taken from the subarachnoid space near vasospastic arteries disclosed concentrations of Hb as high as 30 g/dL (concentration of Hb inside a RBC is 35 g/dL). There did appear to be a correlation between concentration of Hb near the blood vessel and presence of VSP. White et al analyzed contractile response of canine BA *in vitro* to CSF obtained from patients with SAH.[276]

There was no correlation between vasoconstriction and concentration of OxyHb in lumbar CSF. White concluded OxyHb was not an important cause of VSP. The narrow range of concentrations of OxyHb in tested CSF may have prevented a correlation from being observed. In addition, lumbar CSF does not accurately reflect the concentration of OxyHb (or of other spasmogens) next to vasospastic arteries which are often encased in blood clot containing far higher concentrations of OxyHb. Oxyhemoglobin may not be able to exert all of its damaging effects *in vitro*. For example, it probably is not converted to bilirubin *in vitro* [286] and its effects on innervation of arteries would be altered *in vitro*.[273] Another limitation of contractility studies *in vitro* is the short duration of exposure of arteries to vasoactive agents. After SAH, potential spasmogens, such as OxyHb, are present for days next to target vessels.

Experiments attempting to correlate CSF vasocontractility *in vitro* with levels of other potential spasmogens such as lipid peroxides, eicosanoids, or endothelin have not been made, although as discussed, their value might be minimal. As well, poor correlations are usually found between lumbar CSF levels of spasmogens and presence of vasospasm in patients.[177] For similar reasons, lumbar CSF levels of prostaglandins and thromboxanes often correlate poorly [30,279] with presence and severity of VSP and their concentrations in lumbar CSF are too low to elicit maximal contractions of cerebral arteries.[280]

Vasoconstrictors and Vasospasm

Vasospasm could begin as smooth muscle contraction or vasoconstriction but due either to the prolonged and severe nature of contraction or to contraction developing along mechanisms which do not occur normally in smooth muscle, vasoconstriction could, in some cases, become temporarily irreversible.[18,41,141] Alternative theories supposing that proliferative changes in arterial walls or other pathologic structural changes in vasospastic arteries are crucial to prolonged arterial narrowing are reviewed below.

Agonists which constrict human arteries include amines, vasoactive polypeptides, larger proteins such as OxyHb, eicosanoids, lipid peroxides, and various other chemicals, such as uridine triphosphate and KCl.[277,278,280] White felt that amines, polypeptides, uridine triphosphate, and KCl were unlikely to be mediators of VSP either because they produce only phasic contraction of isolated human arteries, because they are only weak constrictors, because they are not present in CSF in adequate amounts during the time of VSP or finally, because antagonists of contractions induced by these chemicals do not reverse VSP or prevent xanthochromic CSF from contracting arteries.[280] He concluded prime candidates for spasmogens included Hb derivatives, plasmin, fibrin degradation products, and arachidonic acid metabolites (including eicosanoids). Evidence for plasmin is poor; it is present in CSF in only small amounts after SAH [61,275] and it is cleared fairly rapidly.[293] Furthermore, intracisternal plasmin prevented post-subarachnoid hemorrhage vasculopathy in pigs.[5] Efficacy of plasminogen activator in preventing VSP eliminates fibrin degradation products as a cause of VSP.[60,61] To the list, however, could be added bilirubin [46,144] and endothelin.[182] Studies of effects of Hb solutions, eicosanoids, lipid peroxides, bilirubin, and endothelin on cerebral arteries both *in vitro* and *in vivo* are therefore relevant to discussion of the cause of vasospasm.

Hemoglobin

Hemoglobin Studies *In Vitro:* Hemoglobin contracts isolated smooth muscle cells and cerebral arteries of several different animal species *in vitro*.[11,12,38,40,41,70-72,74,75,93,99,153-155,157,169,173,175,190,221,239,242,243,246,249,252,271,272] Fujii and Fujitsu reported that OxyHb contracted smooth muscle cells cultured from rat aorta.[70] Ultrastructural changes, including vacuolation, cell degeneration, and loss of internal cell structure were noted after 24 hours of exposure. They used RBC cytosol as OxyHb solution, which would also contain stromal proteins and other RBC cytosol proteins, although these other components are of doubtful significance. Steele and

colleagues used electrophysiology to study smooth muscle cells isolated from rat cerebral arteries.[221] A solution containing OxyHb and small amounts of MetHb contracted cells and increased calcium-dependent potassium currents. Cells died rapidly after exposure. Pure MetHb solution did not cause any of these effects.

Cook et al demonstrated that Hb causes slowly developing and long-lasting contraction of dog cerebral arteries, rabbit ear arteries, and rat stomach fundus *in vitro*.[40] They used a solution of Hb containing 20% OxyHb. Asano and associates performed similar experiments on dog BA *in vitro* and found both OxyHb and MetHb caused dose-dependent contractions.[11,199] Maximum contraction induced by OxyHb was 72.4% ± 2.5% of reference contraction whereas MetHb caused only 22.8% ± 1.6% contraction. Other investigators have also found that OxyHb is a much more potent vasoconstrictor than MetHb.[157,242,246,272]

Systemic and cerebral arteries have differing pharmacologic properties [41]; they also vary in sensitivity to OxyHb.[239,242,246] Tanishima applied pure human OxyHb to basilar, coronary, femoral, and mesenteric arteries from dogs.[242] Dose-dependent contraction of BA occurred but systemic vessels were not affected by OxyHb. Toda and coworkers found OxyHb caused mild contraction of canine mesenteric, coronary, and renal arteries but maximal responses in these arteries were less than 50% of those produced in cerebral arteries.[246] They also reported that differences in responses to OxyHb of cerebral and systemic arteries are greater in primates than in lower mammals.[249]

There are important species differences in sensitivity of cerebral vessels to Hb. Wellum et al found human OxyHb to be a more potent constrictor of dog and rabbit BA than of human BA.[271] Poor responsiveness of human arteries was attributed to post-mortem change. Fujiwara and colleagues reported that OxyHb caused more vigorous constriction of dog BA than rabbit BA *in vitro*.[74] Conflicting data have been reported on effects of Hb-containing solutions on guinea pig BA, emphasizing the difficulty in comparing results using different experimental preparations.[73,160] Tanishima found human BA did not contract in response to OxyHb but that it did respond to noradrenaline.[243] Okamoto and coworkers investigated responses of fresh human, monkey and canine cerebral arteries to erythrocyte hemolysate.[175] Proximal segments of arteries were more sensitive to hemolysate than distal segments. Post-mortem changes affected responses to various vasoconstrictors to different degrees, suggesting one explanation for reports of poor responsiveness of human BA to OxyHb.[243]

Hemoglobin Studies *In Vivo*: Many studies have investigated short-term effects of Hb applied to cerebral arteries exposed *in situ*.[29,33,68,100,104,105,167-169,172,218,219] In 1975, Chokyu exposed rat BA *in situ* and demonstrated a 30% constriction due to topical application of hemolysate of erythrocytes and a 19% constriction due to pure OxyHb.[33] Sonobe and Suzuki applied OxyHb and MetHb to exposed cat BA.[218,219] Oxyhemoglobin reduced BA diameter to 37.3% ± 8.5% of control whereas MetHb showed no spasmogenicity (1.4% ± 3.8%). Kamiyama et al [105] and others [167] have reported similar results. Fox reported that Hb did not contract canine BA exposed *in situ* but it is unclear if the solution used contained OxyHb.[68]

Most of the above experiments confirm that OxyHb causes vasoconstriction. Pure MetHb is probably inert but presence of small amounts of OxyHb will render a solution vasoactive. Wellum et al [271,273] and others [280] have expressed concern that OxyHb is not a strong enough vasoconstrictor to cause severe VSP. However, if OxyHb is responsible for VSP then short-term experiments *in vitro* would not replicate conditions *in vivo* where arteries are exposed to high concentrations of OxyHb for many days. Concentrations of OxyHb used in most *in vitro* and short-term *in vivo* studies ranges from 10^{-8} to 10^{-2} M with maximal contraction developing at concentrations equal to and above 10^{-3} M, depending on the preparation.[11,12,40,75,153,173,175,242,243,246,271,273] Measurements of Hb concentrations in subarachnoid hematomas [169] suggest adequate amounts of OxyHb are present near vasospastic arteries for a long enough time to produce maximum contraction.

The issue of duration of exposure to spasmogen has been addressed in experimental models where changes in arterial diameter were assessed over many days. Miyaoka et al reported that OxyHb injected into the cisterna magna of dogs caused marked VSP of BA which lasted over two weeks.[146] Arterial narrowing lasting 14 days following a single injection is surprising, particularly since VSP resolves one week after single injection of whole blood into the dog cisterna magna.[282] Single intracisternal injections of low doses of OxyHb have been given to small numbers of dogs [161,162,293] and baboons [23] with variable results but, unfortunately, single injections do not produce a model which is similar to the human condition where OxyHb is present adjacent to cerebral arteries for days.[169]

Mayberg and colleagues developed a piglet model of SAH which kept high concentrations of Hb or various blood components adjacent to right MCAs for 10 days.[142] Changes in arterial lumen area were measured using morphometric analysis of fixed arterial sections. Placement of 400 mg of pure porcine Hb (roughly 15% OxyHb) resulted in a significant decrease in lumen area which was equal to that caused by placement of RBC cytosol containing a similar amount of Hb. Degree of VSP correlated with amount of Hb remaining along the MCA at day 10. Although whole blood caused a greater decrease in lumen area than did Hb, this was because the former was associated with more residual clot-containing Hb at day 10. Therefore, all vasoactivity of whole blood could be accounted for by Hb.

Studies of How OxyHb Causes Vasoconstriction: Antagonists of receptor-operated vasoconstrictors, including atropine, methysergide, cinanserin, ketanserin, phenoxybenzamine, phentolamine, mepyramine, chlorpheniramine, propranolol, salbutamol, angiotensin sarcosine alanine, theodur, and quinine have consistently failed to reverse OxyHb-induced contractions of cerebral arteries *in vitro* and *in vivo*.[40,100,104,106,107,153,157,167-169,172,173,178,180,181,242,246]

Drugs reported to relax smooth muscle contracted with OxyHb include papaverine, Ca^{2+}-channel antagonists, and some inhibitors of eicosanoid synthesis.[40,104,106,107,168,178,181,242] Eicosanoids are discussed in the next section. Although Ca^{2+}-channel antagonists partly reverse OxyHb-induced contraction of cerebral vessels *in vitro*,[40,242] these drugs do not dilate monkey [55,163] or human cerebral arteries [8,188] which are vasospastic. Kajikawa et al found that after exposing rat BA *in situ* to barium chloride for 3 hours, papaverine became unable to dilate the BA, providing evidence that duration of contraction bears upon ability of pharmacologic antagonists to relax smooth muscle.[104,168]

Oxyhemoglobin acutely increases intracellular inositol polyphosphates which are second messengers involved in smooth muscle contraction.[260] There are few other reports investigating the intracellular mechanism of action of OxyHb.

Eicosanoids

There are species differences in sensitivities to various eicosanoids but TXA_2, $PGF_{2\alpha}$, and PGE_2 are potent constrictors of human cerebral arteries.[245,261,265] After SAH in monkeys and dogs, vasospastic vessels synthesize less PGI_2 and more vasoconstricting PGE_2.[136,165,202,250,265] No TXB_2 was detected in dog arteries [136] but vasospastic monkey arteries had the same levels of TXB_2 as control arteries.[165] Decreased vasodilator influence from PGI_2, combined with endothelial damage [140] with adherence of platelets,[95] vascular thrombosis,[122,147] and release of vasoconstricting prostaglandins and TXA_2, could contribute to VSP.[136,165,249] Effects of OxyHb on vessel wall eicosanoid synthesis *in vivo* have been investigated by Tokoro.[250] Injection of OxyHb into dog cisterna magna decreased PGI_2 levels in BA 4 and 7 days after injection. There was no change in arterial wall TXA_2. Studies show that following SAH in humans and dogs, CSF contains elevated levels of $PGF_{2\alpha}$ and generally other prostaglandins such as PGE_2 and PGD_2.[30,82,193,194,207,208,264,265,279] Increased levels of vasoconstricting prostaglandins have, in some instances, correlated with presence of VSP in patients after SAH.[194,207] Others have not found such correlations.[278,279]

Experiments *in vitro* using bioassay systems to measure eicosanoids suggest OxyHb alters release of these chemicals from the arterial wall,[174,249] potentially accounting for changes seen after SAH. Okamoto and colleagues assayed superperfusate from canine pial arteries on various smooth muscle preparations.[174] Hemolyzed erythrocytes were applied to a bath containing a dog pial artery. Superperfusate from this bath contracted rat stomach strips, relaxed dog coronary arteries, and had no effect on dog ileal strips. Pretreatment of canine pial artery with indomethacin abolished or attenuated responses of assay organs to superperfusate, suggesting that hemolysate, and presumably OxyHb, causes release of vasoactive PGs from dog pial arteries.

Toda found indomethacin and diphloretin phosphate attenuated OxyHb-induced contractions of dog and monkey BA and MCA.[246,249] This contraction was endothelium-dependent. Using a bioassay system of dog coronary artery, he concluded that contractions induced by OxyHb were dependent on a mechanism related to endothelium but not involving endothelium-derived relaxing factor (EDRF). He suggested prostaglandins released from endothelium were important in this effect and demonstrated that RBC hemolysate interfered with PGI_2 synthesis in dog MCA *in vitro*.[247]

Effects of inhibitors of prostaglandin and thromboxane synthesis on experimental and human VSP have been equivocal.[34,75,76,203,230,241,283] Studies of OxyHb-induced dog BA contraction *in vitro* show indomethacin and aspirin, inhibitors of cyclooxygenase, have no effect on [71,72,157,242] or only partially prevent such contractions.[40,73,246,249] Thromboxane synthetase inhibitors were also ineffective in preventing OxyHb from contracting dog and guinea pig arteries.[160,239]

Thus, although OxyHb affects vessel wall eicosanoid production and could account for alteration in CSF levels of these substances after SAH, inhibitors of synthesis of prostaglandins and thromboxanes do not consistently prevent VSP. If OxyHb acts at multiple sites in the vessel wall (for example, direct action on smooth muscle cells by release of free radicals and production of lipid peroxides, combined with release of eicosanoids, endothelium-derived relaxing factor, and endothelin from endothelium) then antagonizing only one of these systems might not completely reverse contraction. Another possibility is other spasmogens, which act through non-eicosanoid mediated paths, are important. Furthermore, other eicosanoids, such as leukotrienes and their intermediary metabolites, the hydroperoxyeicosatetraenoic acids, have not been thoroughly investigated.[102,181,213,261,292] Shimizu et al found lipoxygenase activity was elevated in dog BA after SAH and this resulted in increased LTB_4 and LTC_4 in BA itself but in no detectable leukotrienes in CSF.[213] Yokota and colleagues reported similar results.[292]

Free Radicals

Misra and Fridovich observed that shark OxyHb spontaneously autoxidizes to MetHb, releasing superoxide anion radical ($O_2^{\cdot -}$).[145] Mammalian OxyHb also autoxidizes, although more slowly.[274] Spectrophotometric studies show that after SAH, hemolyzing subarachnoid erythrocytes release OxyHb which autoxidizes to MetHb, potentially releasing $O_2^{\cdot -}$ into the subarachnoid space.[48,128,129,251,254,262] Superoxide anion radical, in conjuction with iron in Hb, has been postulated to initiate and propagate lipid peroxidation by the iron-catalyzed Haber-Weiss reaction.[11,197,199] Hemoglobin has not been unequivocally shown to act as a biological Fenton reagent but it does stimulate decomposition of lipid peroxides.[85] Lipid peroxides are known to cause vasoconstriction and structural damage to cerebral arteries both *in vitro*[125,126] and *in vivo*.[200,201]

There is additional evidence for a role of lipid peroxidation, and presumably OxyHb, in genesis of VSP. Vasoactivity of blood incubated *in vitro* correlates with its content of thiobarbituric-acid reactive substances and with concentration of OxyHb,[11,199,200] although fractionation of the incubated fluid shows vasoactivity resides in substances with a molecular weight equal to OxyHb and not to lipid peroxides.[218,219] Substances that react with thiobarbituric acid include lipid peroxides.

Concentrations of lipid peroxides in CSF of patients with SAH correlate with VSP.[11,199,200,205,266] A potent inhibitor of iron-dependent lipid peroxidation, U74006F, significantly diminished VSP in a primate model of SAH.[222] Despite questions about the exact mechanism of initiation of lipid peroxidation after SAH,[84,85,197] detection of 5-hydroxy eicosatetranoic acid in CSF after SAH proves that lipid peroxidation (at least by enzymatic pathways) occurs.[229] Although OxyHb probably plays a pivotal role in propagating lipid peroxidation after SAH, it has not been shown *in vivo* that the process is essential for development of VSP. This would require evidence that lipid peroxidation preceeds development of VSP since the arterial wall damage which accompanies VSP would produce lipid peroxides.[58] Furthermore, since VSP correlates best with large volumes of subarachnoid blood,[66] lipid peroxides in CSF, whether or not their production is related to VSP, would be expected to be elevated in association with VSP.

The culprit in generating free radicals after SAH has usually been considered to be OxyHb. Free radicals, however, are known to be produced by other physiologic and pathologic mechanisms, including xanthine-xanthine oxidase reactions, inflammation and phagocytosis, eicosanoid synthesis, the electron transport chain, during thrombosis, and by a variety of other cellular enzymes.[58,257] The subarachnoid space is inflamed after SAH and free radicals derived from this source could be involved in VSP. There are alterations in eicosanoid synthesis after SAH [136,165,202,250,265] and several investigators have found platelet aggregation in vasospastic arteries.[95] Kim et al dismissed a role for xanthine oxidase in VSP based on their findings in dogs where allopurinol prevented increased CSF urate associated with SAH but did not prevent VSP or morphologic damage to BA.[118]

Effects of free radical scavenging enzymes on vasoactivity of OxyHb on cerebral arteries *in vitro* have been reported.[71,72,168,172,173,272] Kamiyama and coworkers found superoxide dismutase (SOD), catalase, and 1,4-diazabicyclo-2,2,2-octane were effective inhibitors of OxyHb-induced contraction of cat BA exposed *in situ*.[105] Application of an $O_2^{\cdot-}$-generating system to the artery (xanthine and xanthine oxidase) caused vasodilation. Fujita et al contracted dog BA *in vitro* using KO_2 to generate $O_2^{\cdot-}$ in a bath.[71,72] Oxyhemoglobin-induced contractions were blocked by catalase but not by SOD. Wellum et al [272] and others [172,173] found SOD and catalase did not prevent OxyHb from constricting dog BA *in vitro* and exposure of the artery to xanthine and xanthine oxidase did not contract it.

Electrophysiologic studies of isolated rat cerebral artery smooth muscle cells have shown OxyHb activates calcium-dependent potassium currents and causes cell death.[221] Catalase protected cells from OxyHb whereas SOD did not. Xanthine and xanthine oxidase did not cause electrophysiologic changes in cells whereas generation of hydroxyl radical in the bathing solution was damaging. The findings implicate hydroxyl free radical rather than other oxygen free radicals although it may be difficult to implicate single free radical species based on scavenger experiments.[84,85]

Free-radical scavengers, iron chelators, and antioxidants have only infrequently been used to treat VSP.[259,283] Asano and colleagues found AVS (1,2-bis(nicotinamide)-propane) to promptly resolve pre-existing cerebral VSP and to prevent development of VSP in a dog model.[13]

In summary, SOD and catalase alone are poor antagonists of OxyHb-induced cerebral artery contraction. There are several possible reasons for this. Free radical mechanisms may not be important or may not be the sole pathway involved. As mentioned above, multiple sites of action of OxyHb in the vessel wall could obscure results of studies antagonizing only one effect of OxyHb. Superoxide dismutase and catalase may not be able to penetrate arterial walls to locations where damaging free radical reactions occur.[85] Activity of both SOD and a hydrogen peroxide-scavenger may be necessary to prevent genesis of oxygen-derived free radicals since SOD produces hydrogen peroxide which can form hydroxyl free radical in the presence of iron or ferrous proteins.[36,84,257] Catalase or glutathione peroxidase, if present, would

catabolize hydrogen peroxide, preventing this reaction.[257] Hydrogen peroxide [244] can also inhibit SOD.

Endothelium-Dependent Effects

Furchgott and Zawadzki showed vasodilatory action of acetylcholine (ACh) on rabbit aorta was mediated by release of an intermediate substance from endothelial cells.[77] This substance has been called endothelium-derived relaxing factor (EDRF). Other vasodilators relax vascular smooth muscle through this endothelium-dependent mechanism which has been subject to recent review.[10]

After SAH in dogs, vasospastic BA contracted with $PGF_{2\alpha}$ or uridine 5'-triphosphate exhibits impaired relaxation to arginine vasopressin and thrombin.[119,120] Endothelium-dependent relaxation is also inhibited after SAH in rabbits [94,151,152] and monkeys.[108]

Oxyhemoglobin and other ferrous hemoproteins are well known inhibitors of endothelium-dependent relaxation.[10,31,49,74,93,106,107,132,137,138,160,178,223,238,248,252] Martin et al investigated effects of Hb on relaxation of rabbit aortic rings precontracted with phenylephrine.[137,138] Oxyhemoglobin completely blocked endothelium-dependent relaxation induced by ACh whereas MetHb, globin-free ferric porphyrin, alkaline hematin, non-iron containing porphyrin, and protoporphyrin IX had no effects. Methemoglobin slightly inhibited endothelium-dependent relaxation by the calcium ionophore A23187 but had no effect on relaxation caused by glyceryl trinitrate. Oxyhemoglobin substantially blocked relaxations induced by both of these chemicals. Whether or not endothelium-dependent relaxation occurred was found to correlate with levels of cyclic guanosine monophosphate (cGMP) in the aortic ring; OxyHb but not MetHb prevented increased cGMP associated with ACh, A23187, or glyceryl trinitrate.

Fujiwara and coworkers, using rabbit BA, demonstrated that OxyHb blocks endothelium-dependent relaxations induced by ACh and adenosine triphosphate.[74] Blockade of endothelium-dependent relaxation by OxyHb or RBC hemolysate has also been demonstrated in rabbit coronary artery [223] and dog MCA.[10,178] A study *in vivo* of endothelium-dependent relaxation and Hb was reported by Byrne et al.[29] In pigs, intracisternal injection of OxyHb caused acute vasoconstriction of intrathecal arteries. Intracarotid infusion of ACh caused vasodilation before intracisternal injection of OxyHb and vasocontriction afterwards.

Kanamaru et al found xanthochromic CSF of SAH patients to inhibit A23187-induced endothelium-dependent relaxation in dog BA *in vitro*.[106,107] Levels of OxyHb in CSF correlated with degree of inhibition of endothelium-dependent relaxation.

The ability of OxyHb to inhibit endothelium-dependent relaxation suggests it is OxyHb which is responsible for this event after SAH. How OxyHb prevents the action of EDRF has been investigated. Subarachnoid hemorrhage and OxyHb damage arterial endothelium, possibly decreasing EDRF synthesis by endothelial cells.[62,140,170] Kim et al, however, used a bioassay system for EDRF to show that EDRF secretion by vasospastic dog BA was not impaired compared to normal BA.[120] Endothelium-derived relaxing factor may be nitric oxide [10] and Hb binds nitric oxide with an affinity 1500 times higher than its affinity for oxygen.[79] Thus Hb, which presumably does not enter cells, could prevent EDRF, if it is nitric oxide, from entering smooth muscle cells and producing its effects. Alternatively, Hb could disrupt pathways leading to cGMP production within smooth muscle cells.

Effects of OxyHb on EDRF have been assessed using more physiologic systems *in vitro* which isolate the intraluminal and extraluminal aspects of the artery. Hongo et al selectively applied OxyHb to intraluminal and extraluminal aspects of rabbit BA precontracted with serotonin.[93] Acetylcholine-induced endothelium-dependent relaxations were more potently inhibited if the intraluminal aspect of the artery was exposed to OxyHb. Tanaka and Chiba, using dog internal carotid artery and stainless steel cannula insertion method, found extraluminal OxyHb to have no effect on ACh-induced vasodilation whereas intraluminal OxyHb completely abolished vasodilation.[238] These results were interpreted as suggesting that OxyHb impairs EDRF by decreasing

release from endothelial cells and/or by binding to EDRF. Inability of extraluminal OxyHb to inhibit endothelium-dependent relaxation suggest this effect of OxyHb is unimportant in VSP. Toda and colleagues, however, applied OxyHb intra- or extraluminally to dog femoral artery with endothelium.[248] Perfusion fluid flowed onto a bioassay tissue of coronary artery without endothelium. Since OxyHb releases prostaglandins from arteries [174,249] they added indomethacin to the perfusion fluid. Extraluminal OxyHb did decrease activity of EDRF as detected by degree of relaxation of the bioassay tissue precontracted with $PGF_{2\alpha}$. Tsuji and colleagues found that extraluminal OxyHb prevented responses of dog BA to intraluminally-applied drugs which have effects mediated by endothelium, such as acetylcholine, thimerosal, and A23187.[252]

Effects of Hb on endothelium are further complicated by observations that ACh causes transient hyperpolarization and relaxation of rat aorta precontracted by noradrenaline.[31] Hemoglobin prevents relaxation but doesn't alter hyperpolarization. It has been hypothesized that ACh releases EDRF and endothelium-derived hyperpolarizing factor (EDHF) from endothelial cells. Nishiye et al observed similar effects in guinea pig BA.[160]

In addition to EDRF, EDHF, and eicosanoids, endothelial cells synthesize a potent vasoconstrictor peptide, endothelin.[291] Elevated levels of endothelin have been found in CSF after SAH [139,206] and OxyHb augments release of endothelin from cultured bovine cerebral artery endothelial cells.[135] Recent evidence suggests that oxygen-derived free radicals, including $O_2^{\cdot -}$ and hydroxyl free radical, may themselves act as endothelium-derived contracting factors in some blood vessels.[255] This is another potential mechanism of action of OxyHb in causing VSP since OxyHb probably can generate these radical species.

The importance of endothelium in VSP remains unclear, although OxyHb contracts cerebral arteries denuded of endothelium, suggesting endothelin release is not requisite for VSP.[38,52,73,154,160] Further, if endothelium is removed after arteries are contracted with OxyHb, relaxation does not occur.[252]

Bilirubin Formation

In 1949, Jackson injected bilirubin into the cisterna magna of dogs, producing severe inflammatory reactions with fever, malaise, and increased CSF protein and leucocytes.[101] The magnitude of reaction was comparable to that induced by OxyHb and he felt one of these two agents was the major irritant involved in hemogenic meningitis. Angiography was not done.

Experiments by Chokyu [33] using exposed rat BA and by Ohmoto et al [167] using exposed cat BA found bilirubin had no effect on blood vessel diameter. In these studies, bilirubin was applied to arteries for minutes only. Duff and colleagues applied bilirubin solutions to exposed BA of 5 cats and 2 baboons.[46] After 4 hours there was progressive vasoconstriction and electron microscopy of the arteries showed swelling of endothelial cells, degeneration of axons and varicosities in the adventitia, and extensive vacuolation of smooth muscle and endothelial cells.

Miao and Lee prepared bilirubin solutions with deionized water.[144] Applications of bilirubin solution to cat and pig internal carotid arteries and to dog BA caused contractions which were more severe than those induced by norepinephrine or serotonin. Bilirubin also blocked endothelium-dependent relaxation and potentiated norepinephrine-induced contraction.

Although the time course of appearance of bilirubin in CSF after SAH is similar to that of vasospasm [16,128,129] there are several reasons to doubt that bilirubin is an important spasmogen. Bilirubin contaminates CSF in other diseases such as neonatal and obstructive jaundice.[123,124,262] Vasospasm or focal neurologic deficits which might be related to VSP, have been noted only rarely in these conditions.[190] Wahlgren et al [262] found bilirubin concentrations in CSF in patients with obstructive jaundice which were the same as levels causing VSP in the study of Duff and coworkers.[46] Bilirubin, especially when bound to albumin, possesses antioxidant activity and inhibits lipid

peroxidation.[224-226] Furthermore, production of bilirubin in the subarachnoid space is by a limited capacity enzyme system and may result in bilirubin concentrations which are too low to induce significant arterial narrowing after SAH.[263] Further work is needed to define the role of bilirubin in VSP, some of which is provided in this thesis.

Neurogenic Effects

Cerebral arteries receive adrenergic, cholinergic, and peptidergic innervation and possess receptors for other neurotransmitters such as serotonin, α- and β-adrenergic, dopamine, and histamine although the precise role of these nerves and receptors in regulation of cerebrovascular tone is unknown.[50,78,90,183,232,233]

Adventitial nerve endings in cerebral arteries degenerate after experimental SAH [43,44,54,78,90,133,141,170] and this is accompanied by transient loss of catecholamine histofluorescence around cerebral arteries.[43,183] Disappearance of perivascular nerves after SAH, however, did not correlate with VSP in rats [133] and monkeys.[90] Sympathectomy can have no effect on [150] or can alleviate VSP [227] in experimental animals and humans, respectively. Drugs that block α, β, and muscarinic receptors have little effect on cerebral VSP.[283] Denervation of cat cerebral arteries *in vivo* with 6-hydroxydopamine produced pathologic changes in tunica adventitia which resembled those seen after SAH, although the remainder of the vessel wall was normal, suggesting other mechanisms must be involved in VSP.[192]

Svendgaard and coworkers investigated the role of neuropeptides in experimental VSP.[233] They made the unique proposal that blood does not induce VSP by direct action on arteries but that a neural reflex is involved, the afferent arm being substance P-containing trigeminovascular sensory nerves and the efferent arm being central pathways from the brainstem to the median eminence, from which spasmogens were secreted to act on arteries. This would not explain why VSP correlates with location of subarachnoid clot. Further work on neuropeptides will be necessary before their role in VSP can be determined.

Despite contradictory evidence regarding significance of changes in cerebrovascular innervation in relation to VSP, SAH clearly does damage these nerves and OxyHb may be responsible for this degeneration. Okada et al found OxyHb caused disappearance of small-cored vesicles in periadventitial nerve endings of cat BA.[170] Lee and colleagues found erythrocyte hemolysates blocked neurogenic vasodilation and potentiated neurogenic vasoconstriction of dog cerebral arteries *in vitro*.[131] Linnik and coworkers reported that OxyHb, while preventing electrically stimulated neurogenic vasodilation of pig cerebral arteries *in vitro*, did not alter uptake of ^{3}H-choline into arterial nerves.[132] They concluded that ACh nerves were not important in vasodilation.

Pharmacologic studies suggest that OxyHb-induced vasoconstriction is not mediated by perivascular nerves or post-synaptic receptors for known neurotransmitters. Atropine, tetrodotoxin, and adrenergic blockers do not attenuate OxyHb-induced contractions of cerebral arteries *in vitro*.[40,73,157,178,220,242] Iwai and colleagues applied OxyHb to exposed cat BA.[100] Contractions were not blocked by phentolamine, prazosin, or adrenergic denervation.

Available evidence indicates OxyHb damages perivascular nerves but that this damage alone is not responsible for VSP, although inhibition of vasodilator nerves and potentiation of vasoconstricting nerves may create a favorable setting for the development of arterial narrowing after SAH.[132] Few studies *in vivo* have been reported and obviously, results might be different when arteries are *in situ* with anatomically-intact innervation. Effects of OxyHb on peptidergic innervation of cerebral arteries have not been investigated in detail.[232,233]

Synergistic Effects

Oxyhemoglobin accentuates contractile effects of hypoxia,[146,154] serotonin,[94,239] potassium,[160] and fibrin degradation products [99] on cerebral arteries *in vitro*. Contributions that these effects make to VSP have not been defined.

MECHANISM OF ARTERIAL NARROWING

Many reports describe pathologic changes in vasospastic arteries following experimental and human SAH.[3,4,17-19,37,39,56,57,59,62,69,96,111,116,122,141,147,158,171,184,192,198,216,217,235,236,240,290,294,295] In humans dying after SAH, edema of the tunica adventitia and tunica media,[39,96,122,147] media thickening and fibrosis,[122,147,198,216,217] smooth muscle cell necrosis,[96,122,147,198,216,217] fragmentation of internal elastic lamina,[198,216] intimal thickening,[96,122,147,198,216,217] and infiltration of adventitia with inflammatory cells, macrophages, and plasma cells [96,216] have been reported. Eldevik et al studied cerebral arteries from 9 patients who died with VSP after SAH and found no specific pathologic changes in patients who died within 2 weeks of SAH.[51] After 2 weeks, endothelial thickening due to proliferation of myointimal cells has been reported.[96,216] Conway and McDonald found, in 6 patients dying 4 to 60 weeks after SAH, "loosely arranged fibroblastic tissue" which encroached on the arterial lumen.[39] This change was also observed in one patient dying 4 days after SAH. Other investigators have observed intimal fibrosis and thickening more acutely after SAH.[216,217] They have postulated that these changes, rather than vasoconstriction, account for lumen narrowing.[111,184]

Seven to 10 days after experimental SAH, vasospastic arteries often show endothelial cell vacuolation and dense body formation, disruption of intercellular tight junctions, and endothelial cell desquamation as well as tunica media changes including smooth muscle cell vacuolation, dense body formation, and necrosis.[4,17,19,37,56,57,60,62,116,171,192,198,236,240,290,294,295] Occasionally, fragmentation of the internal elastic lamina has been reported.[37,198,236,240] Again, in some models, no pathologic changes are observed.[51,189,209]

Study of vasospastic arteries removed more than 2 weeks after experimental SAH show them to have thickened arterial walls, a change variously attributed to inflammatory cell infiltration, edema, proliferation of myointimal or fibroblastic cells, and collagen deposition in the tunica intima.[4,37,122,147,198,235,236,294,295] Several investigators have reported thickening is due to smooth muscle cell hypertrophy, fibrosis, or inflammatory cell infiltration of tunica media.[37,116,122,147,184,198,294,295] These striking changes have also been observed more shortly after SAH,[37,184] supporting the theory that arterial narrowing after SAH is due to a structural change within the arterial wall, such as fibrosis, inflammation, hypertrophy, or hyperplasia. More specifically, Smith et al have proposed that SAH causes migration of myofibroblasts into the arterial wall tunica media and intima, with their resultant proliferation and deposition of type V collagen.[217] The process has been likened to that occurring in granulating wounds. It was suggested that myofibroblasts do not contract themselves but that they exert traction on the extracellular matrix of the arterial wall, causing lumen narrowing.[289] Further evidence in support of structural theories is the remarkable resistance of spastic human vessels to pharmacologic dilation,[17-19,108,121] the delayed onset of VSP, the failure of spastic arteries to reconstrict after transluminal angioplasty,[92,296] and the evidence of vessel wall stiffening which occurs in VSP.[17-19,108,121]

The structural theory, however, remains controversial for several reasons. First, acute vasoconstriction alone results in convolution of the internal elastic lamina and marked arterial wall thickening [62,80,253] and if it is prolonged, can become pharmacologically irreversible and associated with smooth muscle cell necrosis and endothelial cell degeneration.[104] Intracisternal injection of norepinephrine [3] or 15-hydroperoxy arachidonic acid into dogs results in typical pathologic changes in endothelium and smooth muscle of BA.[201] Second, although most investigators observe degenerative changes in vasospastic arterial walls, some do not [51,189,209] and there is no consensus regarding proliferative changes and timing of their development.[96] Third, arterial wall cross-sectional area should increase if proliferative changes cause lumen narrowing. Arterial wall areas have only been measured in one study and increases large enough to encroach on the lumen were not found.[141] Thus, it remains unclear if VSP results primarily from smooth muscle contraction which, for some reason, damages

smooth muscle, or if subarachnoid blood is toxic to arteries, resulting in luminal narrowing due to inflammation, fibrosis, and endothelial cell proliferation in arterial wall, with smooth muscle contraction being a secondary component of the vasculopathic reaction.

If OxyHb is the sole agent necessary for initiation of VSP, then, regardless of the significance of the changes, it should cause the particular arterial wall changes which have been observed in association with VSP. Okada et al injected OxyHb into feline prepontine cistern.[170] After 24 hours, animals were killed and BA, which were shown by angiography to have 33% decrease in diameter, were examined. Myonecrosis, transformation of nerve endings, invasion of myointimal cells into tunica intima, changes in endothelial cell basement membrane, and detachment of endothelial cells were observed. Ohta and colleagues applied OxyHb to exposed rat BA for 3 hours.[168] Vasospasm resulted and at electron microscopy, endothelial cell craters, blebs, and vacuoles were seen. Smooth muscle cells developed vacuoles, nuclear pyknosis, and mitochondrial degeneration.

Vacuolation and degeneration of cultured rat aortic smooth muscle cells were observed 24 hours after exposure to OxyHb whereas serotonin- and norepinephrine-exposed cells were normal.[70]

Exposure of pig MCAs *in vivo* to Hb for 10 days produced a spectrum of ultrastructural damage including fragmentation of internal elastic lamina, distortion of endothelial cells, smooth-muscle cell pallor, and degeneration of perivascular axons.[141,142] Therefore, OxyHb can produce morphologic changes in cerebral arteries which are indistiguishable from those due to SAH. The biochemical basis, significance, and exact timing and nature of these changes are unclear.

SUMMARY

There is evidence that release of OxyHb from lysing subarachnoid erythrocytes is a key mechanism responsible for VSP. Oxyhemoglobin is present in high concentrations in CSF during VSP. Erythrocytes are the component of blood necessary for VSP to develop and the most vasoactive substance within the RBC is OxyHb. The role of OxyHb, however, has not been well investigated in a long-term study *in vivo*. There is also conflicting data about the vasoactivity of MetHb. The role of other spasmogens in VSP, particularly bilirubin, lipid peroxides, eicosanoids, and endothelin, has not been adequately studied.

Oxyhemoglobin has many effects on cerebral vasculature which could contribute to VSP, including alteration of release of blood vessel eicosanoids, initiation of free-radical induced vascular damage, inhibition of endothelium-dependent relaxation, enhancement of release of endothelin from endothelial cells, metabolism to bilirubin, and damage to perivascular nerves. Contributions of each of these effects to VSP *in vivo* have not been investigated.

In addition, there is still controversy regarding mechanisms of lumen narrowing during VSP and regarding the contribution of pathologic changes in vasospastic arteries to development of lumen narrowing, arterial wall stiffness, and temporary irreversibility of VSP. Finally, biochemistry of changes in arterial smooth muscle, endothelium, and extracellular matrix occurring during VSP are largely unexplored.

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CHAPTER TWO [a]
ETIOLOGY OF CEREBRAL VASOSPASM IN MONKEYS

SUMMARY

A primate model was used to see if oxyhemoglobin (OxyHb), methemoglobin (MetHb), and bilirubin are likely to be responsible for cerebral vasospasm (VSP) following subarachnoid hemorrhage (SAH). Forty cynomolgus monkeys were randomly assigned to one of five groups. On day 0, each animal underwent cerebral angiography followed by right craniectomy and placement of an Ommaya reservoir with its catheter adjacent to the right middle cerebral artery (MCA). Animals received intrathecal injections twice a day for 6 days, with one of the following solutions: 1. mock cerebrospinal fluid (CSF), 2. OxyHb, 3. MetHb, 4. bilirubin, or 5. supernatant fluid from an incubated mixture of autologous blood and mock CSF. On day 7, angiography was repeated and animals were killed. Cerebrospinal fluid levels of malondialdehyde (MDA), a product of lipid peroxidation, and of free-radical scavenging enzymes were also measured to see if VSP is associated with lipid peroxidation and depletion of free-radical scavenging enzymes. Comparison of angiograms taken on day 0 and on day 7, after the 6 days of intrathecal injections, showed significant VSP of right middle cerebral, anterior cerebral, and internal carotid arteries in groups which received OxyHb or supernatant fluid. There was a smaller reduction in diameter of the same vessels in the bilirubin group which did not reach significance while no effects were observed with MetHb or mock CSF. Electron microscopy of right MCAs gave results consistent with angiography. One monkey injected with OxyHb developed a delayed-onset right MCA infarction. Concentration of MDA was elevated in CSF in association with VSP due to OxyHb and supernatant fluid. Intrathecal injections of MetHb or bilirubin did not increase CSF MDA. Activity of glutathione (GSH) peroxidase in CSF increased significantly after injection of OxyHb and MetHb. There were no significant changes in CSF superoxide dismutase (SOD) activity although there was a trend towards higher activities in animals treated with OxyHb, MetHb, and supernatant fluid. These data suggest that OxyHb causes cerebral VSP following SAH. Oxyhemoglobin-induced VSP is associated with MDA and therefore, lipid peroxidation, in the subarachnoid space. Detection of peroxidation products after injection of OxyHb in the absence of erythrocyte membranes indicates that OxyHb may directly damage cerebral arteries and brain by inducing lipid peroxidation in these structures. Depletion of free-radical scavenging enzymes in CSF did not seem to be necessary for VSP to develop. In fact, there was a tendency for VSP to elevate enzyme activities, as if production of these scavenging enzymes was induced by excess free radical production in the subarachnoid space.

INTRODUCTION

While it is generally believed that cerebral VSP after SAH is produced by a compound or compounds released from subarachnoid clot, the nature of these compounds has not been conclusively established.[9,70,74] There is evidence from studies *in vitro* and *in vivo* that OxyHb can produce a contraction of cerebral arteries which is much more prolonged than that seen with agents such as serotonin.[8,56] Several additional findings support the theory that OxyHb is an important mediator of VSP.[1,3,6,10,16,24,29,32,34,36,38-40,42-46,57,77]

There are several unresolved issues regarding the role of hemoglobin (Hb) in vasospasm. For example, whether Hb can cause prolonged arterial narrowing lasting

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[a] A version of this chapter has been accepted for publication. Macdonald, RL, Weir BKA, Runzer TD, Grace MGA, Findlay JM, Saito K, Cook DA, Mielke BW, Kanamaru K: Etiology of cerebral vasospasm in primates. **J Neurosurg** 1991 (in press).

for days, without development of tachyphylaxis, is uncertain. Most experiments have investigated its effects over hours.[1,6,8,16,17,29,32,34,43,56,61,63,71,74] It is also uncertain whether Hb is the sole agent responsible for VSP, or whether it acts in concert with other substances, such as lipids, coagulation-cascade products or other unidentified chemicals.[1,9,26,27,36,74] There is still some debate about the vasoactive effect of MetHb.[1,4,35,63,71] Hemoglobin is also metabolized to bilirubin, a compound regarded by some workers as a prime candidate for the causative agent in VSP.[9,36]

In addition to these questions, mechanisms responsible for vasoactivity of Hb have not been worked out *in vivo*. One theory suggests OxyHb initiates free radical reactions in subarachnoid clot after SAH, resulting in production of toxic lipid peroxides.[1,51,52] These peroxides then cause arterial narrowing. It is unclear if lipid peroxides in CSF arise from breakdown of erythrocyte membranes, from reaction with lipid in brain or arterial wall, or from both. Vasospasm and peroxidation occurring in a system devoid of erythrocyte membranes would suggest there is lipid peroxidation of arterial wall, supporting a direct free-radical mediated action of OxyHb on vasculature.

To clarify these issues, cerebral arteries were exposed *in vivo* to OxyHb, MetHb, and bilirubin for prolonged periods and any VSP which developed was assessed angiographically and by microscopic examination of affected arteries. Effects of these substances were compared to vasoactivity of substances released into the supernatant fluid of blood incubated *ex vivo*. Levels of MDA, SOD, and GSH peroxidase were measured in CSF to see if VSP caused by these agents is associated with lipid peroxidation and with reduction in CSF defences against free radical reactions.

MATERIALS AND METHODS

Protocol

Forty female cynomolgus monkeys (Macaca fascicularis, Charles River Primate Research Corp., Port Washington, New York) weighing between 2.4 and 4.1 kg were assigned by restricted randomization to one of 5 groups of 8 (Fig. II-1). Animals were given intrathecal injections twice a day for 6 days with one of the following solutions: 1. OxyHb, 2. MetHb, 3. bilirubin, 4. supernatant fluid from an incubated autologous blood - mock CSF mixture, or 5. mock CSF alone. On day 7, angiography was repeated and animals were killed. Prior to each injection, CSF was withdrawn from the Ommaya reservoir and frozen at -60°C. The CSF from days 2, 4, and 6 was analyzed for concentration of MDA and activity of SOD and GSH peroxidase. The CSF from days 1, 3 and 5 was analyzed for content of hemoglobin (Hb), OxyHb, and bilirubin. Five animals in each group were randomly preselected for electron microscopic examination of the cerebral arteries. Four of these monkeys were randomly preselected for injection of horseradish peroxidase (HRP) into the Ommaya reservoir on day 7 in order to evaluate patency of the catheter and intrathecal distribution of the injected solutions. Differences in color of solutions precluded blinding of the experimenter although interpretation of angiograms and data analysis were blinded.

Protocols were evaluated and approved by the Animal Ethics Review Committee of the University of Alberta. Care and surgery of animals were performed according to standards set by the Canadian Council on Animal Care.

Preparation of Solutions

Animals destined to receive injections of supernatant fluid had 12 ml of heparinized (100 U per 10 ml) arterial blood drawn on day 0. This was mixed with 12 ml of mock CSF (Elliott's solution B[11]) and the final solution was gently agitated at 37°C in the dark. For injection, a small volume was removed from the flask and centrifuged at 100 X g for 10 minutes. Two ml of supernatant fluid were withdrawn for injection and the remaining solution, along with 2 ml of mock CSF, was returned to the water bath.

Oxyhemoglobin was prepared from purified crystalline human Hb (Sigma Chemical Company, St. Louis, Missouri) by reduction with a 10-fold molar excess of sodium dithionite followed by dialysis (Spectrapor dialysis tubing, molecular weight cut-off of 12,000 to 14,000 daltons, Spectrum Medical Industries, Inc., Los Angeles, California) over 24 hours with 400 volumes of mock CSF at 4°C.[34] Similarly, MetHb was prepared from human Hb by oxidation with a 1.2-fold molar excess of potassium ferricyanide followed by dialysis. Spectrophotometric analysis on a co-oximeter (Instrumentation Laboratories model 282, Instrumentation Laboratories, Inc., Lexington, Massachusetts) showed the OxyHb solution contained 50% OxyHb and 50% MetHb and the MetHb solution contained over 99% MetHb. Hemoglobin was stored in small aliquots at -80°C for up to 1 month prior to use.

Immediately prior to injection, 2.1 mg of pure bilirubin (Sigma Chemical Company, St. Louis, Missouri) was suspended in mock CSF by shaking. The pH (Fisher Accumet model 610A pH meter, Fisher Scientific Co., Ottawa, Ontario) and osmolarity (Wescor 550 vapor pressure osmometer, Wescor, Inc., Logan, Utah) of bilirubin and Hb solutions were 7.4 and 275 mOsm/L, respectively. Over the 6 days, osmolarity of supernatant fluid ranged from 271 to 292 mOsm/L and pH ranged from 7.6 to 8.1 (see results). Elliott's solution B was found to have the following composition: pH; 7.4, osmolarity; 275 mOsm/L, Na^+; 141 mmol/L, Cl^-; 119 mmol/L, K^+; 3.9 mmol/L, Mg^{2+}; 0.40 mmol/L, Ca^{2+}; 1.32 mmol/L, PO_4^- (inorganic); 0.53 mmol/L, and glucose; 4.3 mmol/L. Sterility of all injected solutions was confirmed periodically.

Studies of Supernatant Fluid

Samples of monkey blood were drawn from 12 animals and placed into 12 sterile tubes containing heparin (100 U per 10 ml). Each sample was mixed with an equal volume of mock CSF and incubated in a shaking water bath at 37°C in the dark, exactly as for set up of supernatant fluid for injection. Twice each day, supernatant fluid was removed from 3 mixtures, as described above, and analyzed immediately for pH (Fisher Accumet model 610A pH meter, Fisher Scientific Co., Ottawa, Ontario), osmolarity (Wescor 550 vapor pressure osmometer, Wescor Inc., Logan Utah), and content of Hb, OxyHb, and MetHb (measured by co-oximetry). Supernatant fluid from 6 mixtures was removed once a day and stored at -80°C for future analysis for concentrations of protein, bilirubin, MDA, and activities of GSH peroxidase and SOD.

Protein determinations were made by Lowry assay performed in triplicate on each sample.[31] A detergent stock solution containing 10% deoxycholic acid and 10% Tween-20 was prepared to add to samples to inactivate any viral particles which might be present. To 0.9 ml sample, 0.1 ml detergent solution was added and the resulting mixture diluted 2 to 4 times with distilled H_2O. Fifty μL of diluted sample was added to 450 μL distilled H_2O (10-fold dilution) and mixed thoroughly. Sixty μL of the resulting mixture was placed in a cuvette with 170 μL distilled H_2O and 2.5 ml solution A (consisting of a mixture of 2 ml 2% $CuSO_4$, 2 ml 4% potassium tartrate, and 98 ml Na_2CO_3 [3% in 0.1 M NaOH]) and allowed to stand for 10 minutes at room temperature. Folin reagent (Folin-Ciocalteau reagent, diluted 2-fold, Sigma Chemical Co., St. Louis, Missouri), 250 μL was added to the cuvette. The solution was mixed for 10 seconds and allowed to sit for 30 minutes at room temperature after which time absorbance was measured at 650 nm on a Beckman DB-G grating spectrophotometer (Beckman Instruments, Fullerton, California). A standard line was generated using different concentrations of bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri) with and without adding detergent. Blanks (distilled H_2O as sample) were also tested.

Bilirubin assays were carried out under red light. Samples were centrifuged at 6500 X g for 5 minutes. Eighty μL of fluid was removed and mixed with 240 μL methanol, 480 μL chloroform, and 8 μL 2 M H_2SO_4. The solution was vortexed for 30 seconds and allowed to stand at room temperature for 15 minutes. After vortexing again for 30 seconds, 240 μL methanol and 350 μL water were added and the mixture

vortexed for 3 seconds and centrifuged at 6500 X g for 2 minutes. Upper aqueous phase was aspirated off and 450 μL of lower chloroform phase was removed and dried under a stream of N_2 at room temperature in an N-Evap analytical evaporator (Organomation Associates, Inc., Northborough, Massachusetts). Dried precipitates were stored for up to 7 days at -20°C. Prior to analysis by high pressure liquid chromatography (HPLC), samples were reconstituted in 40 to 100 μL of HPLC mobile phase (24.15 g dioctylamine in 1 L methanol [HPLC grade] with 8.9 ml glacial acetic acid adjusted to pH 7.0 to 7.2 with dioctylamine, filtered through a 0.22 μm nylon filter and made up to contain 3.5% water). Mobile phase was eluted isocratically at 1 ml/min through a Supelcosil 25 cm x 4.6 mm LC-18-S reversed phase column preceeded by a Supelcosil 2 cm x 4.6 mm LC-18-S guard column (Supelco Canada Inc., Oakville, Ontario) mounted on a Gilson liquid chromatograph (Gilson Medical Electronics, Middleton, Wisconson). Detection was based on absorption at 454 nm on a Gilson HV Holochrome UV spectrophotometer. Bilirubin was quantitated by integrating peak area using a Gilson 704 version 2.1 HPLC system manager software package and an Apple IIa computer with a Gilson model 620 datamodule and comparing to a standard curve generated with bilirubin standards. Lower limit of detection was a concentration of bilirubin of 1 μM in the original sample.

Bilirubin standards were prepared using crystalline bovine bilirubin (Sigma Chemical Co., St. Louis, Missouri). Bilirubin was dissolved at 2 mg/ml in 0.1 N NaOH which was purged with N_2 for 30 minutes at room temperature. The solution was vortexed and immediately added to bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri) at 13.2 mg/ml in 5 mM phosphate buffer, pH 7.4 and 4°C to give a bilirubin/albumin ratio of 3:1. This mixture was diluted 20 times in 5 mM phosphate buffer, pH 7.4 at 4°C. Volumes of 80 μL were removed and extracted, dried, and stored as described above. Standards were reconstituted in 53.3 to 800 μL of HPLC mobile phase to give standards of 300 ng to 20 ng per 20 μL.

Dose Considerations

Experiments at the University of Alberta using the cynomolgus monkey model of SAH have shown that a 5 ml blood clot placed in the subarachnoid space causes VSP.[14] This clot contains approximately 600 mg of Hb. Complete metabolism of the Hb would yield 25 mg of bilirubin. Oxyhemoglobin was administered as 12 injections of 1 ml of a 7.8 X 10^{-4} M solution of Hb containing 50% OxyHb (total dose of 300 mg of OxyHb and 300 mg of MetHb). Methemoglobin was administered as 12 injections of 1 ml of a 7.8 X 10^{-4} M solution of Hb containing over 99% MetHb (total dose of 600 mg of MetHb). Hemoglobin was slowly released into the incubated supernatant fluid so that monkeys injected with supernatant fluid received 550 mg of Hb (300 mg as OxyHb) over the course of 12 injections of 2 ml each (see results). Therefore, monkeys in the OxyHb and supernatant fluid groups received the same total amount of OxyHb. Bilirubin was injected as 12 divided doses of 2.1 mg each (total dose of 25 mg of bilirubin). In the mock CSF group, each injection consisted of 1 ml of Elliott's solution B.

Day 0: Baseline Assessment and Cerebral Angiography

Prior to handling, the animals were sedated with ketamine HCl, 6 to 10 mg/kg intramuscularly. Each monkey was weighed and underwent baseline physical assessment. They were endotracheally intubated and ventilated on a 2:1 mixture of N_2O:O_2 administered by a variable-phase animal respirator (Harvard Apparatus, Inc., Millis, Massachusetts). End-tidal PCO_2 was monitored continuously and adjusted to approximately 40 mm Hg (Patient Monitor 78356A, Hewlett Packard, Federal Republic of Germany). Paralysis (gallamine, 2 mg/kg intravenously) and anaethesia (fentanyl, 1 μg/kg intravenously) were induced. A heating pad was used to keep body temperature at 37°C as monitored by a rectal thermometer (Tele-thermometer, Yellow Springs Instrument Co., Yellow Springs, Ohio). Procaine penicillin, 100,000 IU/kg and atropine, 0.6 mg/kg were administered intramuscularly.

Using sterile technique and magnification, the femoral artery on either side was dissected and catheterized with a sigmoid-tip, radiopaque, 5-F polyethylene catheter. Under fluoroscopic control, the catheter was advanced into the communis artery, from which both common carotid arteries arise in the cynomolgus monkey. It was connected via a three-way stopcock to a pressure transducer for measurement of blood pressure and heart rate (Statham P23dB pressure transducer, Statham Instrument Co., Oxnard, California). Blood pressure and heart rate were continously recorded on a Beckman Dynograph R611 eight-channel recorder (Beckman Instruments, Inc., Fullerton, California). A single anteroposterior, arterial-phase cerebral angiogram was obtained by injecting 12 ml of iothalamate meglumine at 200 psi via a Cordis injector (Cordis Corp., Miami, Florida). Exposure factors were uniform (70 kV, 1.6 mAs) and a magnification control standard was used in each radiograph for correction to constant magnification.

Operative Procedure

Immediately after angiography, anaesthesia was supplemented with pentobarbital, 25 mg/kg intravenously, and additional gallamine as necessary. Ten mls of 20% mannitol was administered intravenously and ventilation was increased to lower the end-tidal PCO_2 to 30 mm Hg. The head was turned left and fixed in three-point pin fixation. A right frontotemporal craniectomy was made, centered on the pterion. Dura mater was opened and the frontal lobe was elevated and the temporal lobe depressed, exposing the ipsilateral optic nerve and internal carotid artery. Under the operating microscope, arachnoid membrane over precommunicating segment of the anterior cerebral artery (ACA), sphenoidal segment of the MCA, and intradural internal carotid artery (C_4) was divided, exposing the underlying arteries. Dura mater was carefully closed with a patch of deep temporalis fascia sutured in place with 6-0 silk suture. A silicone tube with multiple side perforations (Pudenz ventricular catheter, American V. Mueller, Chicago, Illinois) was carefully passed through the dural suture line and along the sylvian fissure until it's tip was adjacent to the carotid bifurcation. The catheter was connected to a CSF reservoir (Ommaya CSF reservoir, American V. Mueller, Chicago, Illinois) situated in the subcutaneous space. Temporalis muscle, superficial fascia, and skin were closed in layers with 3-0 silk suture and 3-0 monofilament polyethylene suture (Dermalon; Davis and Geck, New York, New York). Paralysis was reversed with prostigmine (0.07 mg/kg) and atropine (0.02 mg/kg) intravenously and animals were ventilated with 100% O_2 until they were breathing spontaneously. Animals were extubated when their gag reflex returned.

Days 1 to 6: Intrathecal Injections, Clinical Observations

Intrathecal injections began the day following craniectomy. Twice a day for 6 days, monkeys were sedated with ketamine HCl, 6 to 10 mg/kg intramuscularly, and the appropriate solution was injected into the Ommaya reservoir. Prior to injection, up to 2 ml of CSF was withdrawn from the reservoir and stored at -60°C for analysis of Hb and bilirubin levels. Animals were observed carefully for food and water intake, general activity level, and for evidence of infection or focal neurologic deficit.

Day 7: Repeat Cerebral Angiography and Killing

On day 7, a cerebral angiogram was obtained with animals intubated, ventilated, and anaesthetized under the same conditions as on day 0. In four animals in each group HRP, 80 mg/kg dissolved in Elliott's solution B, was injected into the Ommaya reservoir 15 minutes prior to killing. An overdose of pentobarbital (50 mg/kg) was injected intravenously and animals were exsanguinated. Five animals in each group underwent perfusion-fixation by opening the chest and insertion of a left ventricular cannula, clamping the descending aorta, and opening the right atrium. Circulating blood was washed out with 0.5 L of 0.9% NaCl under 110 mm Hg pressure, followed by 0.5 L of fixative solution (3% glutaraldehyde and 1% paraformaldehyde in Millonig's buffer, 0.12 M, pH 7.4) at 4°C.

Pathological Examination

The brain was removed and examined to ensure the catheter was not impinging on cerebral arteries. The entire circle of Willis was dissected free and in animals which received HRP, arteries were incubated for 10 minutes in a saturated solution of 3,3'-diaminobenzidine (25 mg 3,3'-diaminobenzidine in 50 ml 50 mM Tris-HCl buffer, pH 7.6, and containing 0.01% H_2O_2).[19] Specimens were photographed to show which vessels stained dark brown with HRP reaction products. Cerebral arteries were placed in glutaraldehyde-paraformaldehyde fixative at 4°C until sectioning. Each MCA was divided into 4 or 5 equal portions. One piece was submitted for scanning electron microscopy (SEM, Model 505, N.V. Phillips Gloelampenfabrieken, Eindhoven, The Netherlands) and remaining segments were submitted for transmission electron microscopy (TEM, Hitachi H-7000, Hitachi, Tokyo, Japan). Samples for SEM were post-fixed with 1% OsO_4 in 0.07 M Millonig's buffer at room temperature for 1 hour, washed in distilled water, dehydrated in a graded series of ethanol solutions, immersed in absolute ethanol, and critical-point dried with CO_2 (SeeVac, Inc., Critical point dryer, CPD-100, Pittsburgh, Pennsylvania). Arterial segments were mounted on stubs and sputter coated with gold (Edwards high-vacuum model S150B sputter coater, West Sussex, England) and examined at 15 kV. For TEM, specimens were post-fixed and dehydrated as for SEM. They were then embedded in Araldite (Marivac Ltd., Halifax, Nova Scotia). Thin sections were cut using a Reichert-Jung Ultracut ultramicrotome (Reichert-Jung, Wien, Austria), placed on 300-mesh copper grids, stained with uranyl acetate and lead citrate, and viewed at 75 kV.

Brains were photographed and sectioned coronally and sections from 3 monkeys in each group were stained with hematoxylin and eosin and examined by light microscopy.

Measurement of Hemoglobin and Bilirubin in CSF

Total Hb and percentage of OxyHb and metHb were measured in CSF using co-oximetry. High concentrations of Hb were present in some CSF samples and it was suspected that this would interfere with determination of bilirubin levels by spectrophotometry. Solutions containing various concentrations of Hb and of bilirubin were prepared and bilirubin concentration determined by Jendrassik-Grof analysis[18] (Parallel Analyzer, American Monitor Corp., Indianapolis, Indiana). Pronounced interference was observed (see results) and this method was abandoned and HPLC was used to measure CSF bilirubin by the method described above.

MDA, SOD, and GSH Peroxidase Assays

Malondialdehyde was detected using HPLC.[5] A Whatman chromasorb C1B 5 micron ODS column (4.6 x 150 mm) preceeded by a 50 x 4.6 mm Whatman 5 micron ODS guard column, was eluted isocratically at 1 ml/min with 14% (v/v) acetonitrile in 50 mM myristyltrimethylammonium bromide and 1 mM sodium phosphate (pH 6.4) at room temperature on a Waters series 3B liquid chromatograph. Detection was by absorbance at 267 nm on a Waters variable wavelength spectrophotometer. Malondialdehyde was quantitated by manual measurement of peak height and comparison to known standards.

Superoxide dismutase activity was measured by direct assay with potassium superoxide.[33] Three ml 50 mM AMP-HCl buffer, 5 μL catalase (Boehringer Mannheim Canada, Ltd., Laval, Quebec), 30 μL 0.3 M potassium cyanide, and 10 to 20 μL of sample were added to a 1 ml cuvette. The cuvette was placed in a spectrophotometer (Beckman Instruments, Fullerton, California) set at 250 nm. Potassium superoxide was dissolved in 25 ml ice-cold 50 mM NaOH and 0.5 mM DTPA. Five to 15 μL were transferred to the cuvette and the reaction was followed with a chart recorder (Houston Instruments Omnigraph 3000 recorder, Austin, Texas). Addition of KO_2 was repeated twice. Blanks with sample replaced by H_2O and standards of SOD from erythrocyte hemolysates were also run. Calculation of SOD activity was by estimation of half life

of disproportionation of superoxide. One unit of activity was defined as the activity that disproportionates superoxide at a rate of 0.1 per second.

Activity of GSH peroxidase was measured as described by Wendel.[72] One hundred μL each of buffer, GSH reductase, GSH, and nicotinamide adenine dinucleotide, respectively were transferred to a 1 ml cuvette. Assay solution, 500 μL, was added and the mixture incubated at 37°C for 10 minutes in the spectrophotometer in order to assess the spontaneous rate of reduction of NADPH. The reaction was started by addition of 100 μL of 12 mM cumene hydroperoxide and followed at 366 nm. For samples containing Hb, correction for pseudoperoxidase activity of hemoproteins was made.[20] Hemoglobin concentration was determined and samples were diluted to contain 3 mg Hb per ml. From this solution, 1 ml was mixed with 0.5 ml transformation solution (4.5 mM KCN, 0.45 mM $K_3[Fe(CN)_6]$ adjusted with 0.25 M KH_2PO_4 to pH 7.0). After 5 minutes, 500 μL of the transformed solution was assayed as above. A unit of GSH peroxidase activity was defined as that required to consume 90% of the GSH within 1 minute.

Radiologic Assessment and Data Analysis

Cerebral arteries were measured 6 times with a calibrated optical micrometer, and a mean value obtained. The following arteries were measured bilaterally: extradural internal carotid (C_3), C_4, ACA, MCA, mid-basilar (BA), and vertebral (VA).

All data were coded, entered into a computer, and edited. Physiologic and angiographic data within groups between day 0 and day 7 were compared by paired t-test. Intergroup comparisons of these parameters at day 0 and at day 7 were made by analysis of variance followed by Scheffe's multiple comparison procedure. Total Hb and OxyHb levels in CSF were compared by a Kruskal-Wallis test. Levels of bilirubin in CSF were compared by analysis of variance followed by Scheffe's multiple comparison procedure. Mean MDA concentrations were compared by a Kruskal-Wallis test. Activities of GSH peroxidase and SOD were compared between groups for each time period by analysis of variance followed by Scheffe's multiple comparison procedure. The level of significance for all tests of comparison was $p < 0.05$.

RESULTS

Comparison between groups at day 0 and day 7 revealed no significant differences in body weight, mean arterial blood pressure, heart rate and PCO_2 (Table II-1). The only significant change in these variables within groups between day 0 and day 7 was a decrease in average weight of animals receiving supernatant fluid.

Clinical Status

Five monkeys receiving supernatant fluid developed lethargy, anorexia, and signs of meningeal irritation during the course of injections (Table II-2). Similar signs developed in 5 animals receiving OxyHb, 3 animals receiving MetHb, and one monkey in the bilirubin group. All animals treated with mock CSF remained well. No delayed-onset neurologic deficits were noted although the moribund condition of one monkey in the OxyHb group may have masked a focal neurologic deficit.

Cerebral Vasospasm

For the group receiving supernatant fluid, between day 0 and day 7, there was significant reduction in vessel calibre in right C_3 (22% ± 2%, $p < 0.005$), C_4 (17% ± 6%, $p < 0.05$), ACA (31% ± 10%, $p < 0.05$), and MCA (26% ± 7%, $p < 0.05$). After injection of OxyHb, significant decreases in arterial diameter were noted in right C_3 (19% ± 2%, $p < 0.05$), C_4 (18% ± 5%, $p < 0.005$), ACA (20% ± 6%, $p < 0.05$), MCA (27% ± 5%, $p < 0.005$), and right and left VA (21% ± 5% in each, $p < 0.05$)(Table II-3, Figs. II-2 to II-7). There were no significant changes in vessel calibre in groups receiving bilirubin, MetHb, and mock CSF, although a consistent but mild degree of arterial narrowing occurred in bilirubin-treated animals.

Pathology

Gross Examination: Mechanical compression of right-sided cerebral arteries by the intrathecal catheter was not observed. In animals injected with solutions of OxyHb and MetHb, the anterior cranial fossae and the right middle fossa were consistently covered in thin brown exudate consisting of precipitated Hb. This staining occasionally extended down the clivus into the posterior fossa. Orange globules of bilirubin were seen throughout the basal cisterns and posterior fossa in all animals which received bilirubin. Five monkeys injected with supernatant fluid had brown exudates coating the skull base.

HRP Studies: All animals given intrathecal injections of HRP on day 7 showed HRP-reaction products throughout vessels of the circle of Willis, including both vertebral arteries.

Electron Microscopy: Scanning electron microscopy showed convolution of endothelium and tunica intima, lumen narrowing, and thickening of vessel wall in right MCAs of animals which had received supernatant fluid or OxyHb (Figs. II-8, II-9). Right MCAs from animals injected with bilirubin showed mild convolutional changes and vessel wall thickening. Right MCAs of animals injected with MetHb and mock CSF were usually normal although one animal in each group had minimal endothelial folding. Transmission electron microscopy revealed occasional endothelial cell vacuolation and frequent smooth muscle cell contraction, vacuolation, and necrosis in arteries from monkeys treated with supernatant fluid and OxyHb (Fig. II-10). Pathology was occasionally seen in endothelial cells of animals treated with bilirubin while smooth muscle cells from these arteries were only mildly and infrequently contracted. Arteries from animals injected with MetHb and mock CSF were normal.

Light Microscopy: Hemosiderin-staining was observed in the right sylvian fissure and over the right temporal and frontal lobes in animals injected with OxyHb and MetHb. There was a subacute inflammatory exudate composed of macrophages, polymorphonuclear leucocytes, and loose fibrous tissue in these areas which was more marked and diffuse in OxyHb-injected animals (Table II-2, Fig II-11). Hemosiderin globules were occasionally seen in the posterior fossa meninges. Meninges of animals injected with supernatant fluid showed less hemosiderin staining and less inflammation than seen in animals injected with OxyHb or MetHb. Bilirubin staining and mild subacute inflammation were noted in the bilirubin group. Brains from mock CSF-injected animals were normal.

Sections of brains showed that one monkey (Monkey 31) injected with OxyHb had a cerebral infarct in the right MCA territory which was judged to be 24 hours old (Fig. II-12). This particular monkey had a 50% reduction in right MCA calibre on angiography and had been moribund and not taking fluids prior to day 7 angiography; a focal neurologic deficit was not noted.

Concentrations of Hemoglobin and Bilirubin in CSF

Concentrations of Hb and OxyHb in CSF are shown in Figures II-13 and II-14. While there is substantial variation between samples, it is clear that there is a significant elevation of total Hb at days 3 and 5 in animals treated with OxyHb and MetHb, while the increase also reached significance on day 5 in the group which received supernatant fluid. There were no significant differences at any time in levels of OxyHb, except for a significant increase on day 5 in the group which had received supernatant fluid.

Figure II-15 shows that analysis of bilirubin by Jendrassik-Grof method resulted in marked increases in apparent bilirubin concentration as increasing amounts of Hb were added to solutions containing the same amount of bilirubin. Levels of bilirubin in CSF, determined by HPLC, are shown in Table II-4. The standard line and sample chromatograms are provided (Figs. II-16 to II-19). Wide separation of heme and bilirubin peaks on chromatograms precluded any interference of heme in measurement of bilirubin concentration by HPLC. No significant differences in mean concentration of bilirubin were observed between groups, except, as one might expect, in the group

which had received bilirubin itself. There was some metabolism of Hb to bilirubin as seen by detectable levels of bilirubin in CSF from animals injected with supernatant fluid, OxyHb, and MetHb.

MDA, SOD, and GSH Peroxidase in CSF

Mean concentration of MDA in CSF for each group is shown in Figure II-20. The standard line correlating MDA concentration with peak height (Fig. II-21) and typical chromatograms (Fig. II-22) are provided. Although there were no significant differences between groups, higher amounts of MDA were present in CSF of animals injected with OxyHb and supernatant fluid as compared to CSF from animals injected with MetHb, bilirubin, and mock CSF.

Results of CSF activities of SOD and GSH peroxidase are shown in Figures II-23 and II-24. Representative spectrophotometric absorption lines for SOD and GSH peroxidase assays are provided (Fig. II-25 and II-26). No significant differences were noted between groups at any time period in activity of SOD although there was consistently higher activity in animals injected with OxyHb and MetHb. Supernatant fluid-injected animals also had elevated activity of SOD on day 6, when large amounts of OxyHb were present in the injected solution. Glutathione peroxidase activity increased significantly ($p < 0.05$) in CSF on day 2 in animals treated with OxyHb and MetHb and on day 6 in the OxyHb group ($p < 0.05$), although this latter increase is based on a single CSF sample.

Studies of Supernatant Fluid

Table II-5 contains values for pH, osmolarity and content of Hb, OxyHb, metHb, protein, bilirubin, MDA, and activity of GSH peroxidase and SOD in supernatant fluid from blood-CSF mixtures incubated *in vitro* for 6 days. Osmolarity and pH of supernatant fluid ranged from 273 to 293 mOsm/L and from 7.6 to 8.1, respectively. Assay for bilirubin in supernatant fluid confirmed there was no metabolism of heme to bilirubin *in vitro* and concentrations remained at or below those found in normal serum.

Concentration of protein in supernatant fluid was roughly 30 g/L throughout 6 days of incubation. The standard line for assay showing correlation between concentration of bovine serum albumin with absorbance at 650 nm is presented (Fig. II-27). Protein concentration of OxyHb and MetHb solutions injected were approximately 50 g/L. Concentration of MDA increased to almost twice the day 0 value by day 6. Maximum levels detected were of similar magnitude to amounts found in CSF of animals injected with supernatant fluid.

Activity of SOD in supernatant fluid increased each day. Glutathione peroxidase was detected on the first 3 days of incubation after which activity was diminished or absent.

DISCUSSION

This study shows that of the compounds tested, OxyHb is the agent most likely to be responsible for VSP which occurs after SAH. Oxyhemoglobin and supernatant fluid from incubated erythrocytes both produced a condition which was angiographically and morphologically indistinguishable from VSP caused by presence of whole blood in the subarachnoid space.[14] Bilirubin was substantially less active while MetHb was devoid of vasospastic activity.

These results are similar to those of Mayberg et al who used a model of SAH in piglets to assess the relative contributions of different components of the red blood cell (RBC) to VSP.[35] They applied whole blood, Hb, RBC membranes, and RBC cytosol to the MCA of piglets for 10 days. Hemoglobin caused VSP which was associated with pathologic changes in the arterial wall. The authors concluded that all vasospastic activity of whole blood and of RBC cytosol could be accounted for by Hb. The solution of Hb they used was a mixture of OxyHb and MetHb.

Others have attempted to induce VSP by single injections of putative spasmogens.[4,38,39,77] Boullin et al gave single intracisternal injections of OxyHb to 3 baboons and MetHb to one baboon and found OxyHb caused mild arterial narrowing lasting for 3 days while MetHb was inactive.[4] Other workers have shown a similar phenomenon in dogs.[38,39,77] Unfortunately, a single injection of Hb or other spasmogen does not provide a model which bears similarity to the clinical situation in man. If Hb is released from lysing erthrocytes, it would be expected to occur over a long period. In fact, it has been shown that erythrocytes disappear from the subarachnoid space by hemolysis,[70] liberating OxyHb over the time period during which vasospasm occurs.[3,43] In dogs,[40] cats,[10] and pigs [35] there is evidence that the presence of erythrocytes in the subarachnoid space is necessary for VSP to develop.

Oxyhemoglobin

These observations *in vivo* are also consistent with a substantial body of work conducted in isolated vascular preparations.[1,6,8,15,44-46,56,70,71] Experiments *in vitro* show that OxyHb is the major spasmogen released from RBCs during hemolysis.[38,39,56] Oxyhemoglobin contracts isolated vascular smooth muscle cells and causes ultrastructural damage to the cells.[15] Oxyhemoglobin is a potent constrictor of cerebral arteries both *in vitro* and *in vivo*.[1,6,8,43,56,71]

Previous experiments using the cynomolgus monkey model of surgical placement of formed clot performed at the University of Alberta have produced a 50% reduction in right MCA diameter, a greater degree of vasospasm than observed in this experiment.[14] There are several possible reasons why adherent blood clot causes more severe VSP than OxyHb. In the present experiment, the concentration of OxyHb adjacent to the MCA would not be expected to be as great as the concentration in a subarachnoid hematoma, which has been reported to be as high as 30 g/dL.[43] In addition, Hb released from lysis of RBCs is largely OxyHb, even after cells are incubated *in vitro* for a week [1,56] (Table II-5), while pure OxyHb is unstable and rapidly autoxidizes to MetHb.[73,75] The OxyHb injected intrathecally in this experiment was rapidly converted to MetHb whereas OxyHb injected as part of supernatant fluid seemed to remain as OxyHb for a longer time (Figs. II-13 and II-14). Other substances in RBCs or in whole blood may be responsible for stabilizing OxyHb or for causing cycling of MetHb back to OxyHb.[75] Erythrocytes, and possibly supernatant fluid injected in this experiment, contain MetHb reductase, an enzyme which converts ferric iron of MetHb to ferrous iron found in OxyHb.

Other Spasmogens

Another possibility is that OxyHb acts synergistically with other vasoactive agents, such as lipid peroxides, amphiphiles, or other chemicals.[9,26,27,36,61,74] White et al studied CSF removed from patients with SAH and found no correlation between the amount of OxyHb in CSF and activity of CSF tested on dog cerebral arteries *in vitro*.[74] They concluded that it was necessary to implicate other spasmogens, aside from OxyHb, in development of VSP. The range of concentrations of OxyHb in the CSF was narrow, however, and this may have prevented a significant correlation from being observed. Furthermore, lumbar CSF does not accurately reflect the condition of the subarachnoid space adjacent to vasospastic arteries.[43] In similar experiments, Okwuasaba et al found that in the absence of blood in CSF, no effects were seen, but that even in samples from patients with subdural hematomas, significant activity existed.[46] There was substantial variation in vasospastic activity which did not seem to be a function of presence or absence of spasm in the patient from which CSF was withdrawn. In the present experiment, although concentrations of Hb and OxyHb in supernatant fluid varied with time, by day 6 similar concentrations were being injected into animals receiving OxyHb and supernatant fluid. Similar degrees of VSP were observed, suggesting the activity of supernatant fluid was due solely to its content of OxyHb.

Bilirubin

Bilirubin has been implicated in VSP for a number of years. Jackson found bilirubin caused a severe chemical meningitis when it was injected into the cisterna magna of dogs.[24] Duff et al found bilirubin constricted exposed basilar arteries in cats and baboons.[9] Their theory implicating bilirubin was supported by detection of increasing amounts of bilirubin in blood incubated *in vitro*. Our results, however, show the method used to detect bilirubin (Jendrassik-Grof analysis) gave erroneously high values due to increasing amounts of Hb each day in samples as RBCs lysed. Other investigators agree that living cells probably contain enzymes necessary for breakdown of heme into bilirubin and that such metabolism does not occur *in vitro*.[3,48,55,76]

Miao and Lee observed constriction of cat, pig, and dog cerebral arteries after exposure to bilirubin *in vitro*.[36] In these experiments, arteries were exposed to bilirubin for long periods of time. Other investigators have reported that topical applications of bilirubin solutions to exposed arteries of cats [42] and rats [6] do not produce changes in arterial diameter.

Our results suggest that pathogenesis of OxyHb-induced VSP is not related to its metabolism to bilirubin. Although there was some arterial narrowing in animals injected with bilirubin, this did not reach statistical significance and was not of the magnitude seen with injection of OxyHb. Heme groups of OxyHb and MetHb are broken down into bilirubin at similar rates *in vivo* [76] but while OxyHb is vasoactive, MetHb is not. Thus it is difficult to attribute vasospastic properties of OxyHb to its metabolism to bilirubin. Furthermore, transformation of Hb to bilirubin in the subarachnoid space is limited and may result in concentrations of bilirubin which are too low to induce significant arterial narrowing after SAH.[68] In this study, the bilirubin dose was calculated assuming that complete metabolism of Hb to bilirubin would occur. Although we measured low levels of bilirubin in CSF in animals injected with Hb-containing solutions, only significant amounts of bilirubin were detected in animals injected with bilirubin itself.

There are other reasons to doubt that bilirubin is important in VSP. Albumin, found in CSF, binds bilirubin, and the resulting complex may act as an antioxidant.[59] Binding of bilirubin to albumin profoundly influences its toxicity [9,54,76] although no studies of the concentration of albumin in subarachnoid hematomas have been reported. Binding of other ligands to albumin, such as free fatty acids (which could be present in subarachnoid blood [1,9]) would also influence bilirubin toxicity.[9,41,54,76] Bilirubin is present in CSF for prolonged periods in other diseases such as obstructive jaundice. Wahlgren et al [67] found concentrations of bilirubin in CSF in patients with obstructive jaundice which were the same as levels causing VSP in the study of Duff and coworkers.[9] Patients with hepatic encephalopathy may develop focal neurologic deficits.[47] It is doubtful that these are related to VSP, however, results of investigations such as transcranial Doppler sonography have not been reported in these patients.

Pathogenesis of OxyHb-induced VSP

Pathogenesis of OxyHb-induced VSP may involve autoxidation to MetHb with concomitant release of superoxide free radical,[1,16,17,25,52,53,58,60] inhibition of endothelium-dependent relaxation,[34] augmentation of release of endothelin from arterial endothelial cells,[32] alteration of release of eicosanoids from the arterial wall,[27] and damage to perivascular nerves.[12,29] Since some oxygen-derived free radicals may act as endothelium-derived contracting factors, OxyHb may exert some of its vasoactivity by virture of its ability to release superoxide anion radical and catalyze formation of hydroxyl free radical.[66] Evidence for these effects of OxyHb is based largely on studies *in vitro*. In support of theories invoking free radical mechanisms, these results show OxyHb-induced VSP is associated with elevated MDA levels in CSF, and therefore, lipid peroxidation in the subarachnoid space.

This suggests that erythrocyte membranes, while constituting a potential source of injurious lipid peroxides, are not necessary for VSP to develop. Thus, if lipid

peroxidation is important in VSP, then it is likely that the key free radical reactions are induced in arterial walls by OxyHb. Hemoglobin has been shown to enter arterial walls after SAH.[30] It remains difficult, however, to conclude that free-radical mechanisms mediate the vasospastic activity of OxyHb. Proof of this would require evidence that lipid peroxidation precedes development of OxyHb-induced VSP since vessel wall damage which accompanies VSP [15,44] would be expected to produce lipid peroxides, as most damaged tissues do.[23] Second, inhibition of lipid peroxidation should alleviate VSP. Although U74006F, an inhibitor of iron-dependent lipid peroxidation, significantly diminished VSP in a primate model, substantial arterial narrowing persisted even after treatment with U74006F.[25,58] That OxyHb can contract and subsequently damage cerebrovascular smooth muscle by free radical-mediated mechanisms is, however, consistent with experiments *in vitro*.[15-17,57]

Asano et al [1] first proposed free radical reactions might be important in generating VSP when they noted that OxyHb autooxidizes,[37,73] releasing superoxide anion radical which could be converted to hydroxyl radical by the iron-catalyzed Haber-Weiss reaction.[23,49] Hydroxyl radical could initiate and propagate lipid peroxidation reactions, end-products of which include MDA.[21] Vasospasm was postulated to result from vasoconstriction precipitated by toxic lipid peroxides or possibly by lipoperoxide-induced stimulation of lipoxygenase activity and inhibition of prostacyclin synthetase activity in the arterial wall.[1,52] Direct action of OxyHb on the arterial wall was not ruled out.[1] Indeed, lipid peroxides are vasoconstrictors [26,27,52,53] and they are present in CSF after SAH.[60] High concentrations are associated with VSP [50,52,69] and lipid peroxides have been found within dog arterial walls made vasospastic by OxyHb [64] and by whole blood.[51] Ability of U74006F to significantly reduce VSP following SAH in primates [25,58] and reports of efficacy of antioxidant treatment for VSP [2] suggests lipid peroxidation contributes to VSP.

Although lipid peroxides may be important in VSP and their production is likely through reactions catalyzed by OxyHb or by iron released from it,[23] the present findings indicate that OxyHb or its iron molecules can induce peroxidation in other structures, such as arterial wall. A common suggested mechanism of free radical-induced cell damage is via lipid peroxidation, a process which decreases membrane fluidity, increases membrane leakiness, and damages membrane proteins, including ion channels.[21,23,57,66] Certainly, both direct arterial wall free radical-induced lipid peroxidation and damage due to lipid peroxides produced by erythrocyte membranes could be components of pathogenesis of VSP. Production of eicosanoids, which is by enzymatically-controlled lipid peroxidation,[13] also increases in arterial walls exposed to OxyHb.[45,63]

Free Radical Scavengers and VSP

Lipid peroxidation is normally prevented in aerobic organisms by enzymes including SOD, GSH peroxidase, and catalase as well as by a variety of antioxidant chemicals.[13] Cerebrospinal fluid contains low levels of free radical scavenging enzymes.[22] Inadequate ability of CSF to quench free radicals after SAH has been suggested to be prerequisite for development of lipid peroxidation and VSP. Activities of these enzymes have been measured in CSF after SAH with conflicting results. Our results for SOD activity differ from previous reports which show VSP is associated with diminished CSF SOD activity as compared to activity in SAH patients without VSP.[50,51] We did not introduce whole blood into the subarachnoid space and influx of SOD from blood which initially elevates SOD activity after SAH was not present.

Investigations have shown GSH peroxidase activity increases in CSF after SAH,[50,51] findings which concur with our results. Sakaki and colleagues [50] found no differences in CSF GSH peroxidase activity when comparing patients with and without VSP after SAH. The reason for increased GSH peroxidase activity is unclear although excess hydrogen peroxide, a substrate for GSH peroxidase, could be produced by SOD and by free radical reactions in CSF after SAH and this could induce enzyme synthesis.[22] Decreased SOD activity after SAH has been stated to be evidence that free

radical scavenging ability of CSF is depleted after SAH, allowing development of damaging lipid peroxidation,[50,51,69] a theory apparently at odds with findings related to increased GSH peroxidase activity.

Elevations in free radical scavenging enzyme levels we detected were apparently not enough to prevent lipid peroxidation from occurring. This may be because, despite high CSF levels, enzymes are depleted where free radicals are generated. Hemoglobin penetrates the arterial wall adventitia after SAH, possibly clogging CSF-filled nutrient pores and excluding these enzymes.[30] Sakaki et al found dog arterial walls had diminished GSH peroxidase activity during VSP while CSF activity of GSH peroxidase was significantly elevated.[51] Superoxide dismutase activity was preserved in the arterial wall but depleted in CSF. Even if SOD and GSH peroxidase entered arterial walls, "site-specific" free radical production [23] could still proceed in their presence. In addition, activity of both SOD and GSH peroxidase or catalase may be necessary to prevent damaging free radical reactions since SOD produces hydrogen peroxide which can form hydroxyl radical in the presence of iron or ferrous proteins.[7,22] Catalase or GSH peroxidase, if present, would prevent this reaction by catabolizing hydrogen peroxide. Superoxide dismutase is also inhibited by hydrogen peroxide.[62]

Our results of measurement of SOD activity in blood-CSF mixtures incubated *in vitro* agree with those of Fujita et al.[16,17] Kuwabara and colleagues, however, found SOD activity did not increase in supernatant fluid from blood-CSF mixtures incubated *in vitro*, a result they stated was due to rapid breakdown of SOD in supernatant fluid.[28] Supernatant fluid becomes contaminated with large amounts of Hb as erythrocytes lyse and SOD assays which relay on spectrophotometry in the visible spectrum may give unreliable results.[33] We used ultraviolet spectrophotometry to measure SOD activity by direct assay with potassium superoxide. Other investigators have reported increases in lipid peroxides in blood incubated *in vitro*.[52] Activity of GSH peroxidase has rarely been measured in supernatant fluid from incubated blood-CSF mixtures.

In summary, this study shows that OxyHb causes VSP in monkeys. Once oxidized to MetHb, OxyHb is inactive. Metabolism of the heme group of Hb to bilirubin produces a less vasoactive agent. Vasospasm is probably caused by slow and prolonged release of high levels of OxyHb from hemolyzing subarachnoid erythrocytes. Free radical reactions and lipid peroxidation may be important in the mechanism of OxyHb-induced arterial narrowing. The final biochemical pathways by which increased extracellular concentrations of OxyHb affect the configuration of intracellular contractile proteins await elucidation.

Table II-1: Baseline (Day 0) and Day 7 Values for Physiologic Variables and Angiographic Vessel Diameters, by Group.[a]

	Supernatant Fluid		Oxyhemoglobin		Methemoglobin		Bilirubin		Mock CSF	
Variable	day 0	day 7	day 0	day 7	day 0	day 7	day 0	day 7	day 0	day 7
no. of monkeys	8	8	8	8	8	8	8	8	8	8
body weight (kg)	3.0±0.2	2.7±0.2	3.1±0.5	2.9±0.5	3.0±0.3	2.8±0.2	2.9±0.3	2.7±0.3	2.8±0.3	2.7±0.2
MABP (mmHg)	133±16	112±29	121±16	124±18	114±15	102±28	120±14	115±13	124±23	105±16
heart rate (min^{-1})	202±18	189±31	216±21	201±20	191±18	188±26	201±27	208±33	201±17	192±19
$PaCO_2$ (mmHg)	42±4	39±4	39±2	39±2	39±2	40±1	41±3	41±1	40±2	41±2
Vessel Calibre (mm)										
C3, R	1.46±0.18	1.14±0.19	1.62±0.22	1.31±0.15	1.38±0.12	1.46±0.26	1.67±0.36	1.51±0.40	1.40±0.25	1.50±0.32
C3, L	1.53±0.11	1.23±0.25	1.58±0.28	1.28±0.19	1.36±0.12	1.51±0.25	1.58±0.28	1.55±0.31	1.45±0.19	1.52±0.31
C4, R	1.04±0.15	0.87±0.11	1.09±0.12	0.90±0.09	1.03±0.05	1.02±0.16	1.09±0.13	0.97±0.18	1.03±0.11	1.05±0.16
C4, L	1.07±0.16	0.94±0.15	1.06±0.20	0.99±0.08	1.08±0.07	1.08±0.16	1.06±0.12	1.09±0.13	1.07±0.11	1.09±0.13
A1, R	0.78±0.12	0.54±0.25	0.84±0.12	0.67±0.14	0.77±0.12	0.70±0.07	0.78±0.12	0.67±0.10	0.78±0.16	0.75±0.09
A1, L	0.83±0.16	0.72±0.10	0.85±0.13	0.75±0.20	0.79±0.13	0.84±0.10	0.83±0.11	0.84±0.13	0.77±0.20	0.78±0.08
MCA, R	0.88±0.14	0.65±0.15	0.99±0.10	0.73±0.12	0.83±0.07	0.85±0.14	0.91±0.11	0.79±0.19	0.82±0.12	0.84±0.11
MCA, L	0.85±0.12	0.77±0.09	0.88±0.11	0.79±0.12	0.82±0.10	0.81±0.11	0.85±0.12	0.85±0.10	0.83±0.09	0.89±0.05
BA	1.05±0.24	0.96±0.16	1.17±0.21	1.03±0.18	0.96±0.14	0.92±0.19	1.04±0.27	1.07±0.25	1.08±0.21	1.08±0.24
VA, R	0.89±0.20	0.83±0.11	1.02±0.21	0.80±0.15	0.82±0.05	0.76±0.09	0.98±0.17	0.89±0.23	0.91±0.13	0.98±0.16
VA, L	0.95±0.13	0.84±0.16	1.00±0.21	0.79±0.14	0.81±0.09	0.81±0.14	0.95±0.19	0.88±0.15	0.93±0.12	0.95±0.16

[a] Values are means ± standard deviations. CSF, Cerebrospinal fluid; MABP, mean artery blood pressure; C3, extradural internal carotid artery; C4, intradural internal carotid artery; A1, precommunicating segment of the anterior cerebral artery; MCA, sphenoidal segment of the middle cerebral artery; BA, basilar artery; VA, vertebral artery; R, right; L, left.

Table II-2: Number of Animals in Each Group (n = 8 in Each Group) Which Became Clinically ill During Injections and Degree of Brain Inflammation Observed at Light Microscopy (5 Brains Examined in Each Group)[Graded None (-), Mild (+), or Moderate (++)], by Group.

Group	Ill Animals	Degree of Brain Inflammation
Supernatant Fluid	5	+
Oxyhemoglobin	5	++
Methemoglobin	3	++
Bilirubin	1	+
Mock Cerebrospinal Fluid	0	-

Table II-3: Per Cent Change from Baseline in Right Cerebral Artery Angiographic Diameters, by Group.[a]

	Group				
Vessel	Supernatant Fluid	Oxyhemoglobin	Methemoglobin	Bilirubin	Mock CSF
C3	-22 ± 2 [c]	-19 ± 2 [b]	5 ± 7	-10 ± 5	7 ± 8
C4	-17 ± 6 [b]	-18 ± 5 [c]	-1 ± 5	-11 ± 4	2 ± 3
A1	-31 ± 10 [b]	-20 ± 6 [b]	-9 ± 5	-14 ± 6	-3 ± 3
MCA	-26 ± 7 [b]	-27 ± 5 [c]	3 ± 6	-13 ± 6	2 ± 6
BA	-9 ± 5	-12 ± 6	-4 ± 6	3 ± 4	0 ± 7
VA	-6 ± 5	-21 ± 5 [b]	-7 ± 5	-9 ± 4	9 ± 6

[a] Values are expressed as per cent of change in means ± standard error (n = 8 in each group). C3, extradural internal carotid artery; C4, intradural internal carotid artery; A1, precommunicating segment of the anterior cerebral artery; MCA, sphenoidal segment of the middle cerebral artery; BA, basilar artery; VA, vertebral artery; CSF, cerebrospinal fluid.

[b] $p < 0.05$, paired t-test

[c] $p < 0.005$, paired t-test

Table II-4: Bilirubin Concentration in Aspirated Cerebrospinal Fluid (means ± standard deviations), by Group.

Group	No. of Samples	Bilirubin (μM)
Supernatant fluid	15	4.4 ± 4.6
Oxyhemoglobin	6	8.4 ± 9.0
Methemoglobin	6	4.8 ± 3.1
Bilirubin	14	7.9 ± 5.7 [a]
Mock Cerebrospinal Fluid	16	2.1 ± 1.3

[a] significantly greater than mock cerebrospinal fluid, $p < 0.05$, unpaired t-test.

Table II-5 Osmolarity, pH, Concentrations of Hemoglobin, Oxyhemoglobin, Methemoglobin, Protein, Bilirubin, Malondialdehyde, and Activities of Glutathione Peroxidase and Superoxide Dismutase in Supernatant Fluid from Blood-Mixed Cerebrospinal Fluid Mixtures Incubated *In Vitro* for 6 days. Values are Means ± Standard Deviations (n = 3 for each measurement).

Day	pH	Osmolarity (mOsm/L)	Hemoglobin (g/L)	Oxyhemoglobin (g/L)	Methemoglobin (g/L)	Protein (g/L)	Bilirubin (μM)	Malondialdehyde (nmol/ml)	Superoxide Dismutase (U/ml)	Glutathione Peroxidase (U/ml)
1	7.6 ± 0.1	278 ± 6	1.5 ± 0.6	0.3 ± 0.1	1.2 ± 0.4	28 ± 2	--	0.69 ± 0.57	129 ± 3	1.56 ± 0.80
2	7.7 ± 0.1	274 ± 11	3.5 ± 1.0	2.6 ± 0.1	0.9 ± 0.8	23 ± 1	0.37 ± 0.33	0.71 ± 0.51	173 ± 15	1.49 ± 0.22
3	7.9 ± 0.1	273 ± 6	14.0 ± 6.1	10.0 ± 0.2	4.0 ± 1.1	24 ± 4	0.43 ± 0.41	0.80 ± 0.72	866 ± 57	1.61 ± 1.36
4	8.1 ± 0.2	286 ± 4	30.0 ± 4.8	17.3 ± 1.2	12.7 ± 0.7	27 ± 8	--	0.96 ± 0.88	1413 ± 63	0.16 ± 0.21
5	8.1 ± 0.1	293 ± 8	39.5 ± 1.8	19.9 ± 1.3	19.6 ± 1.4	26 ± 6	0.09 ± 0.09	1.03 ± 0.42	1781 ± 59	0
6	7.7 ± 0.1	280 ± 6	51.1 ± 1.8	23.8 ± 1.4	27.3 ± 1.4	26 ± 7	0.05 ± 0.06	1.05 ± 0.51	1922 ± 42	0

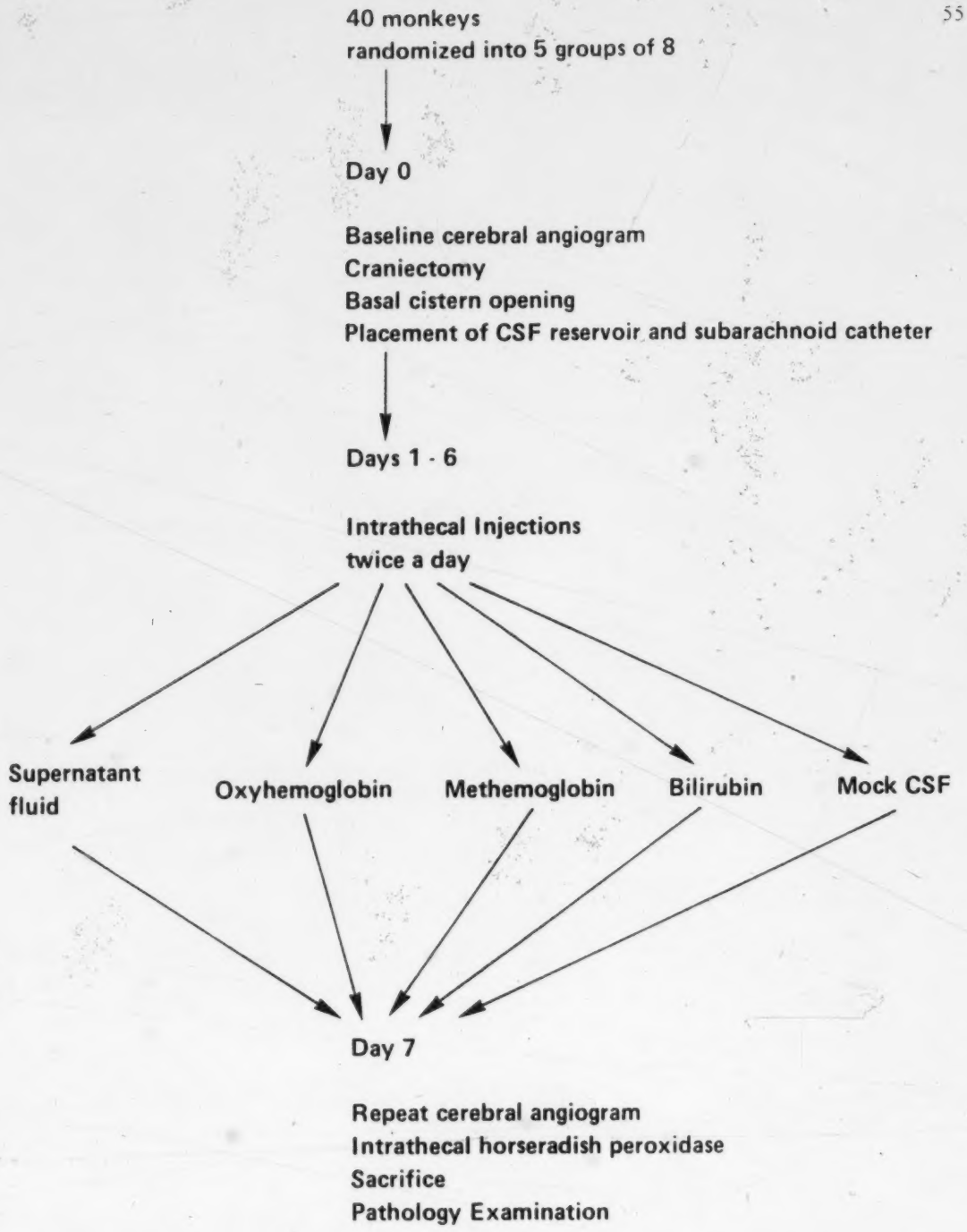

Figure II-1: Flow chart showing experimental design for randomized study of 40 cynomolgus monkeys given intrathecal injections of oxyhemoglobin, methemoglobin, bilirubin, mock cerebrospinal fluid (CSF), or supernatant fluid from incubated blood-mock CSF mixtures.

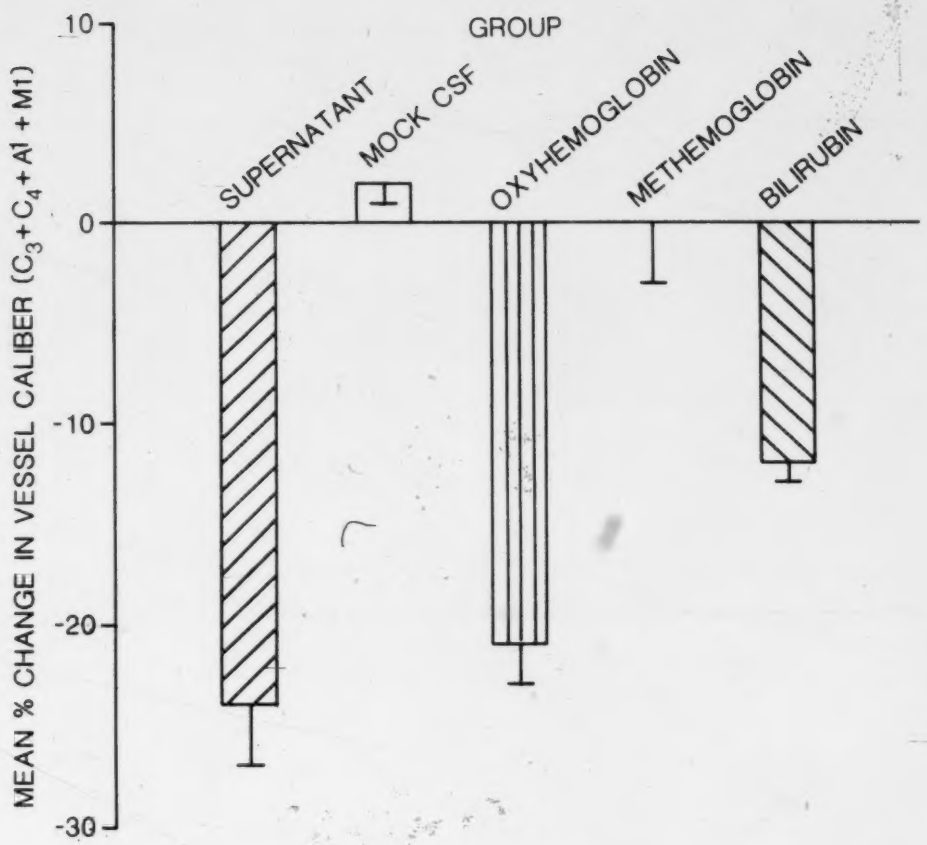

Figure II-2: Effect of multiple intrathecal injections of supernatant fluid, oxyhemoglobin, methemoglobin, bilirubin, or mock cerebrospinal fluid (CSF) on right cerebral artery diameters of monkeys (n = 8 in each group). Bars represent mean per cent change between baseline (day 0) and day 7 in diameter of right internal carotid (C3 and C4), precommunicating anterior cerebral (A1), and sphenoidal middle cerebral (M1) arteries (± standard error).

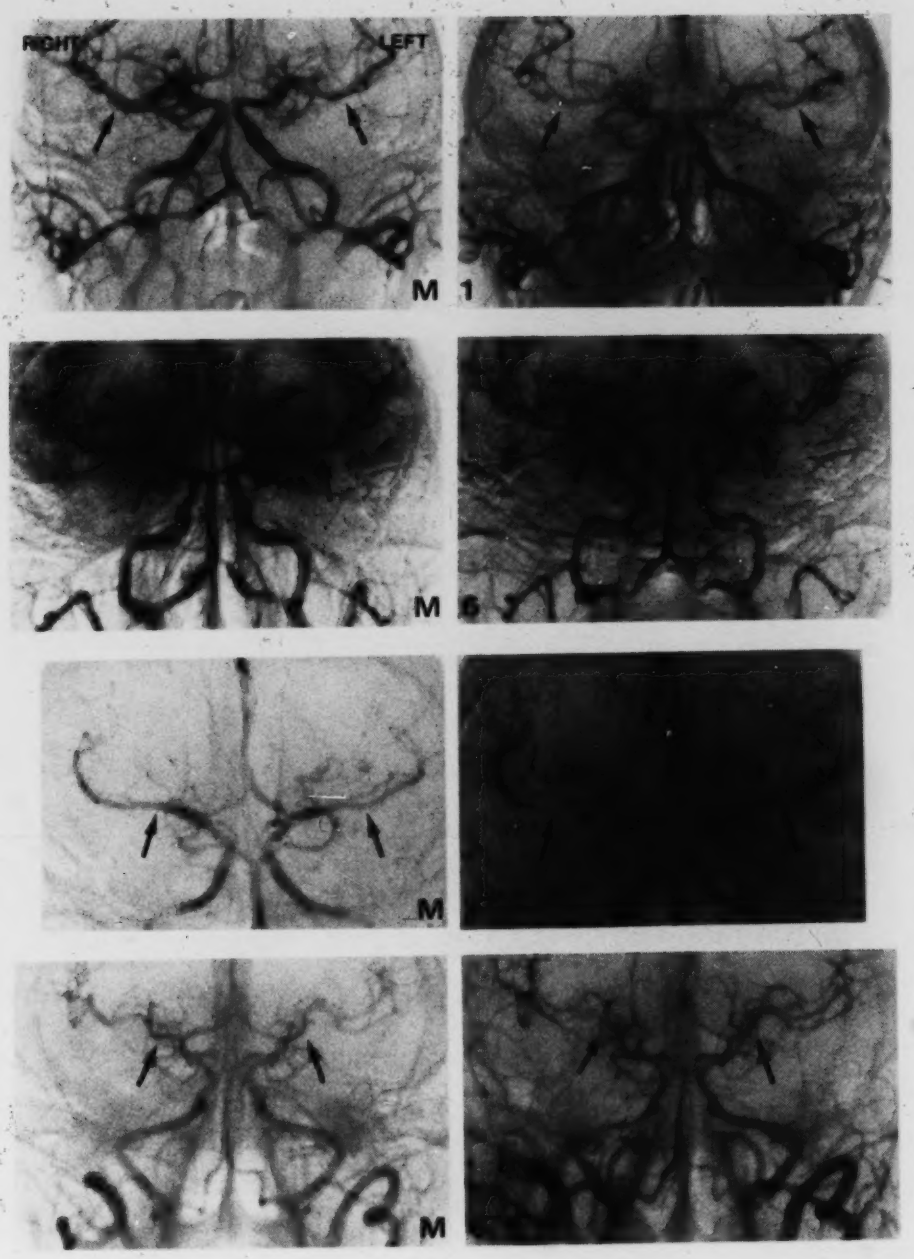

Figure II-3: Baseline (day 0, left) and day 7 (right) angiograms from monkeys injected with supernatant fluid from mixtures of incubated autologous blood and mock cerebrospinal fluid. Comparison of angiography on day 0 and on day 7 shows significant narrowing of right middle cerebral arteries ($p < 0.05$, paired t-test). Arrows point to middle cerebral arteries.

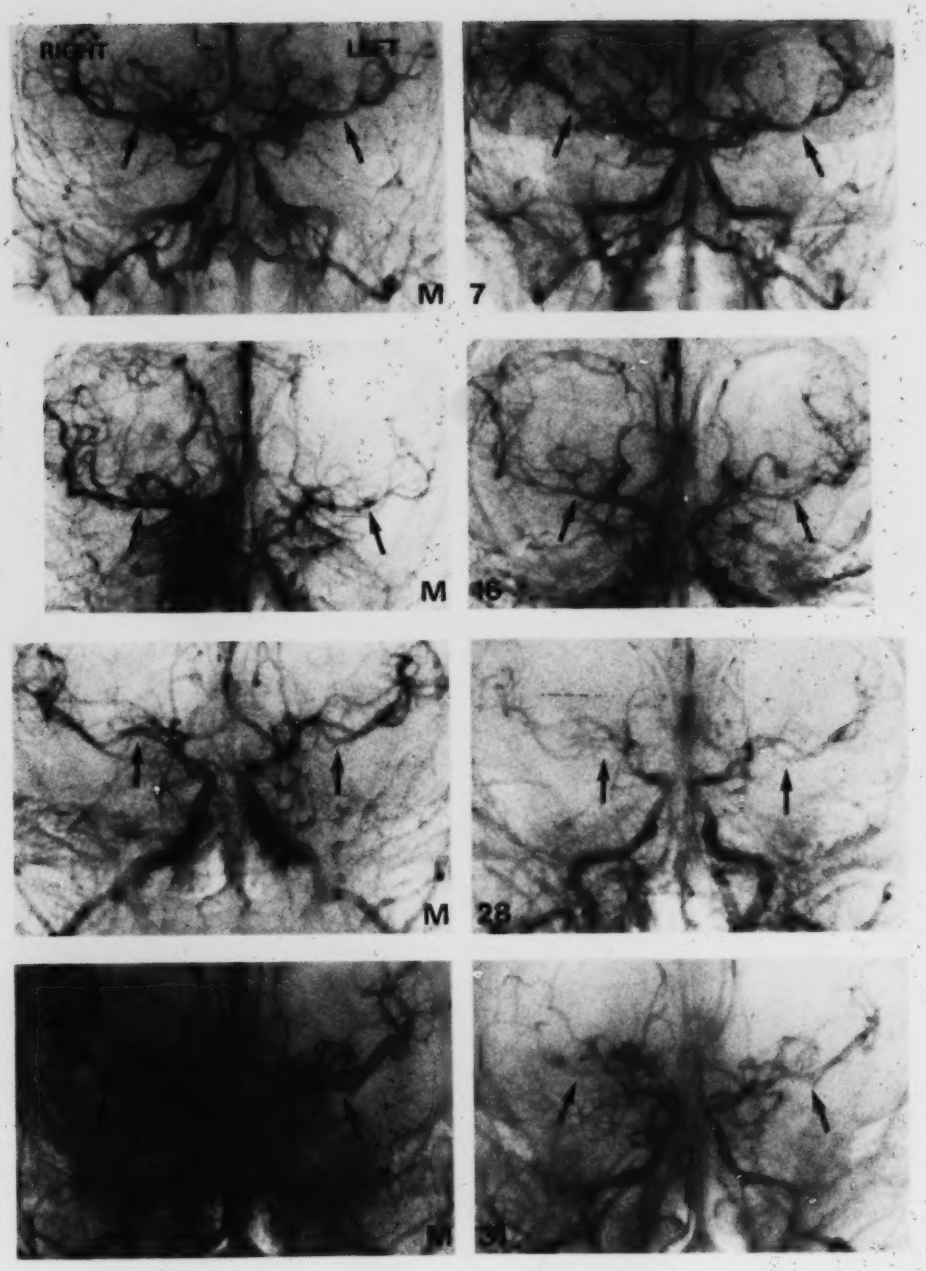

Figure II-4: Baseline (day 0, left) and day 7 (right) angiograms from monkeys injected with oxyhemoglobin. Comparison of angiography on day 0 and day 7 shows significant narrowing of right middle cerebral arteries ($p < 0.05$, paired t-test). Arrows point to middle cerebral arteries.

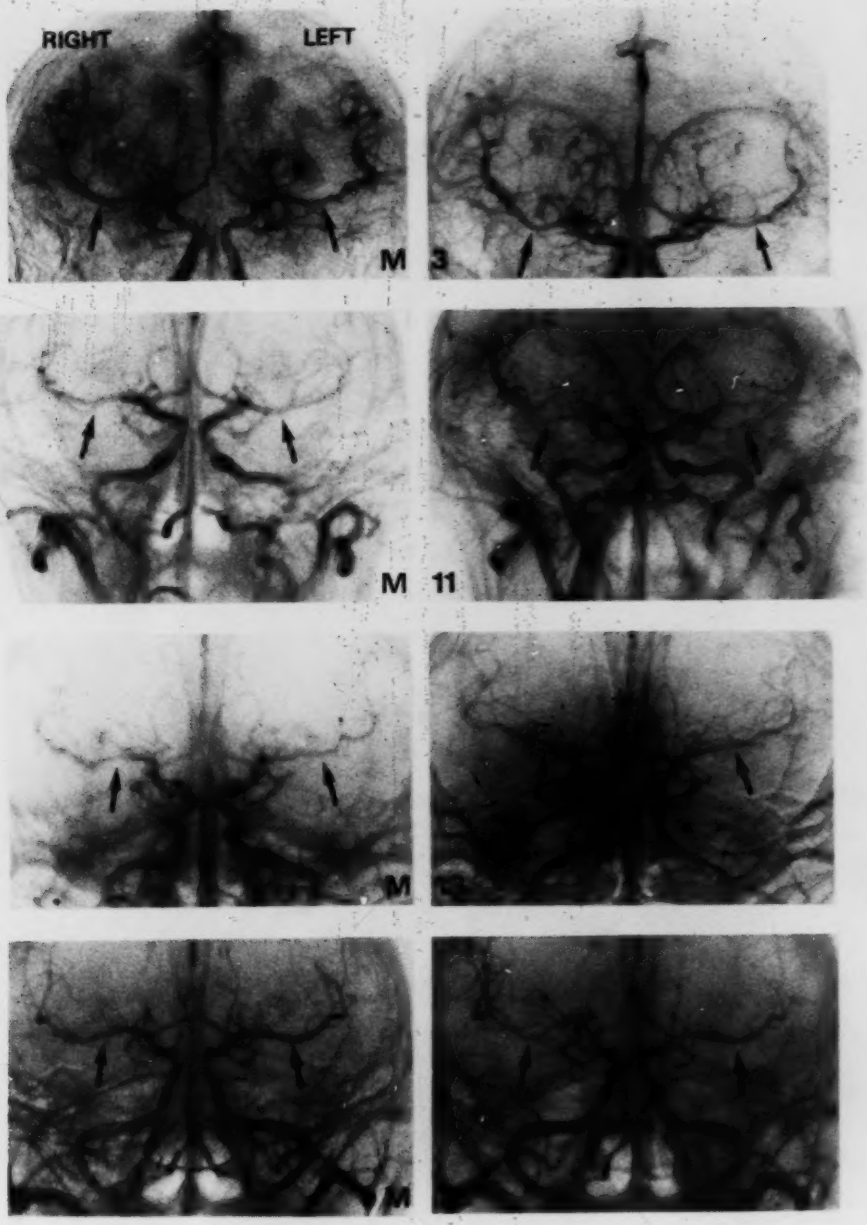

Figure II-5: Baseline (day 0, left) and day 7 (right) angiograms from monkeys injected with bilirubin. In each animal there is mild but insignificant narrowing of the right middle cerebral artery. Arrows point to middle cerebral arteries.

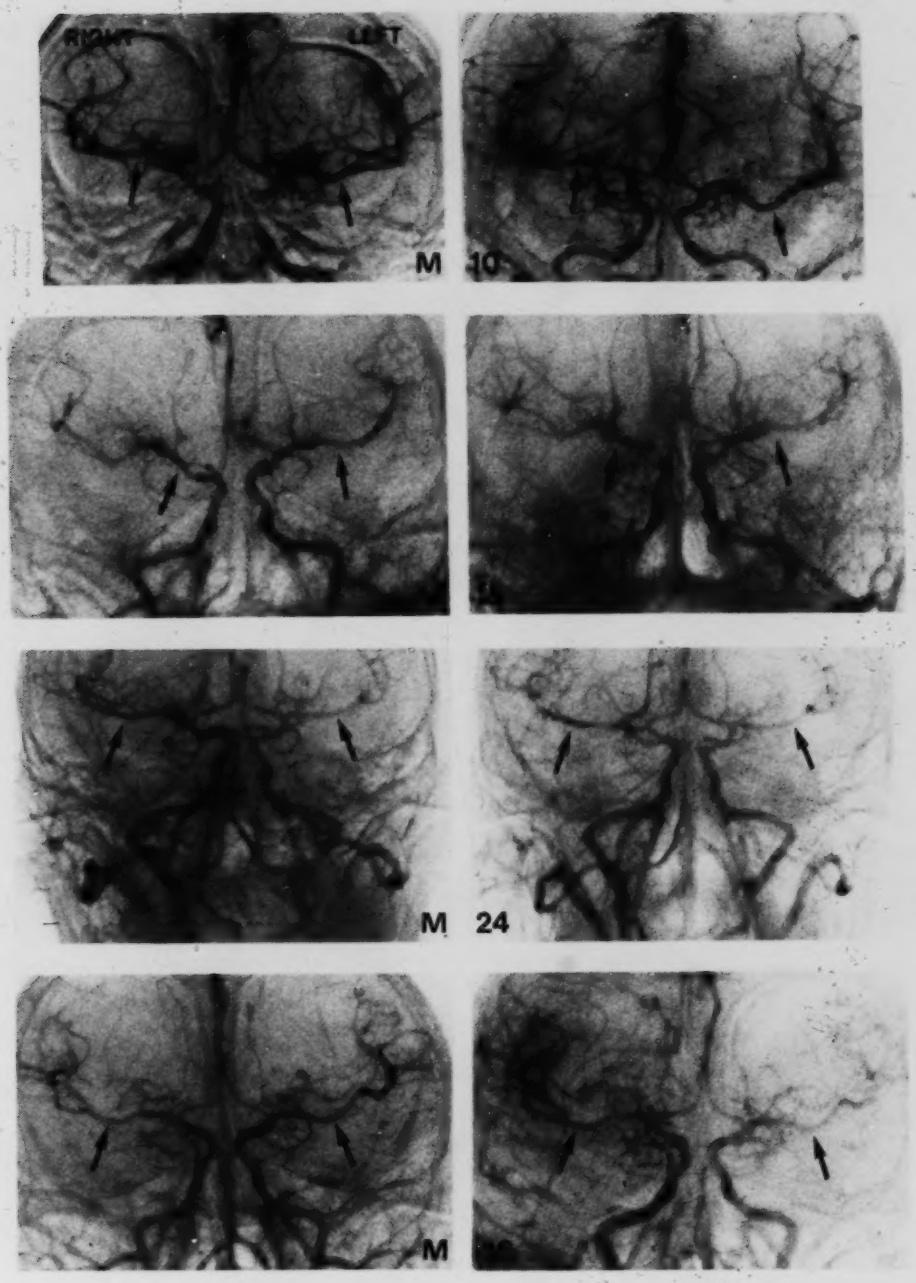

Figure II-6: Baseline (day 0, left) and day 7 (right) angiograms from monkeys injected with methemoglobin. In each animal there is no change over time in diameter of the right middle cerebral artery. Arrows point to middle cerebral arteries.

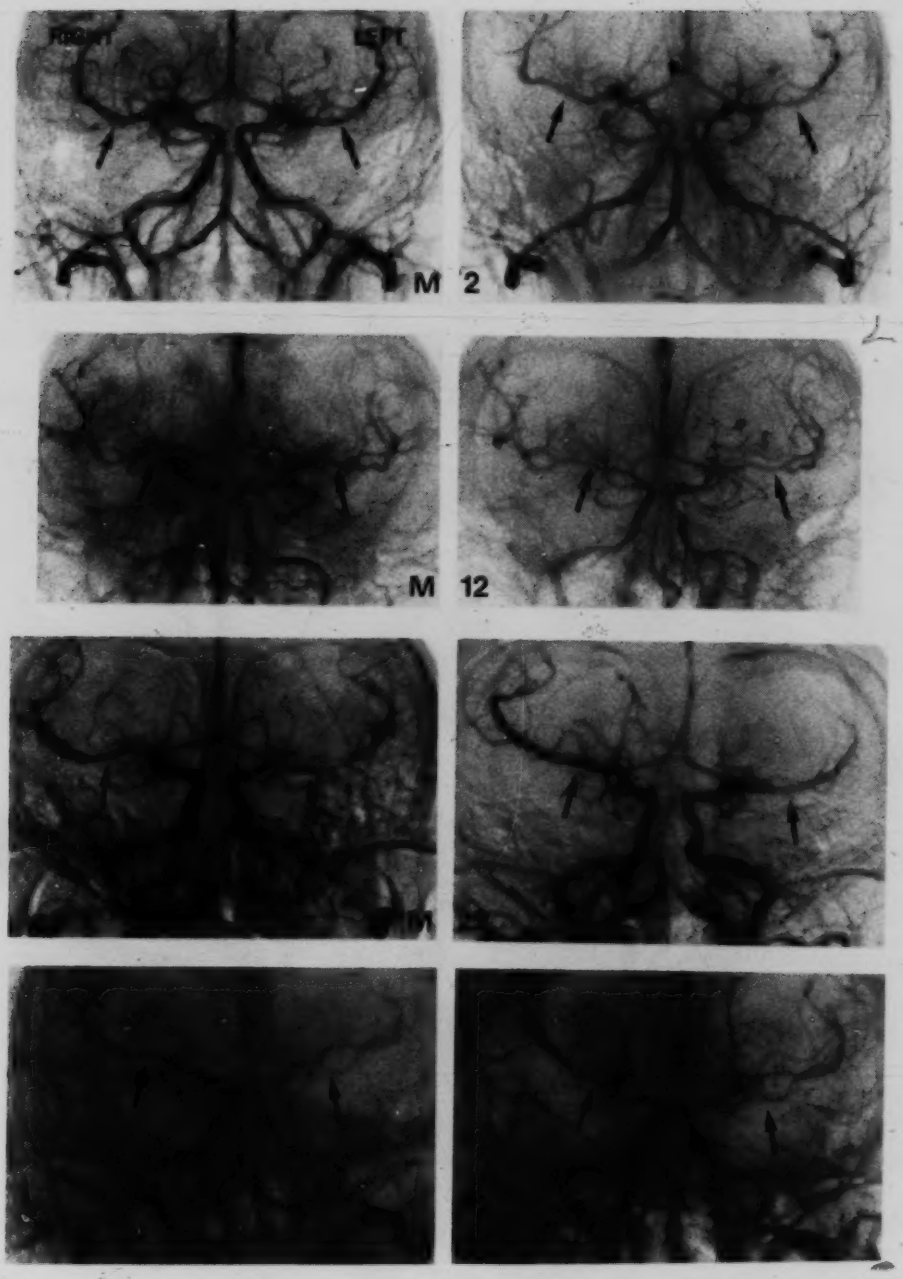

Figure II-7: Baseline (day 0, left) and day 7 (right) angiograms from monkeys injected with mock cerbrospinal fluid. In each animal the right cerebral arteries do not change in calibre between day 0 and day 7. Arrows point to middle cerebral arteriees.

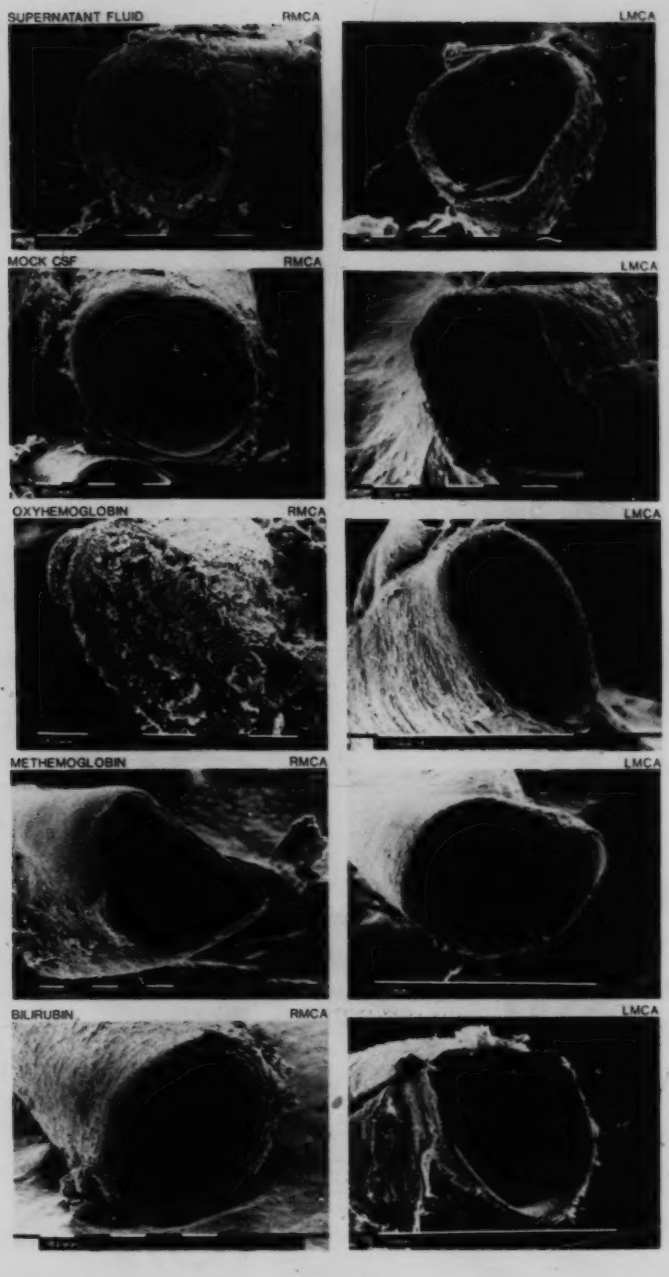

Figure II-8: Scanning electron photomicrographs of right (R, injected side) and left (L) middle cerebral arteries (MCAs) from each group. Scale bars are shown at the bottom of each photograph. All left side vessels are normal. There is vessel wall thickening and convolution of the endothelium in right MCAs from animals injected with supernatant fluid and oxyhemoglobin, in addition to luminal thrombus in the latter. Changes are milder in the MCA of the monkey injected with bilirubin. Right MCAs from monkeys in the methemoglobin and mock cerebrospinal fluid groups are normal. All photomicrographs taken at 15 kV.

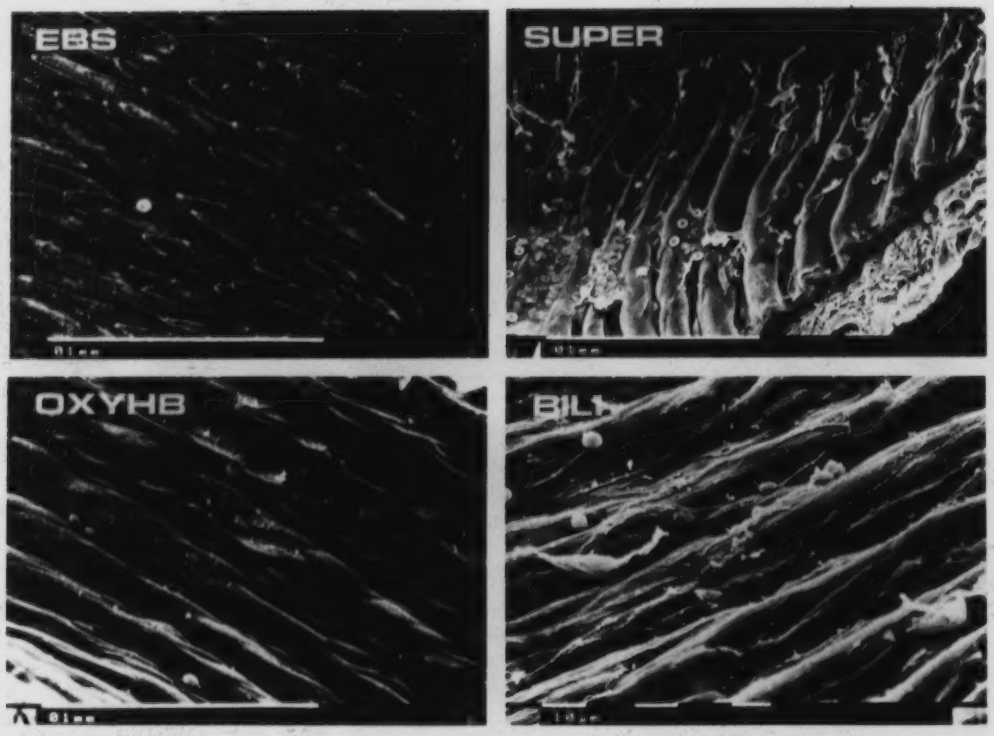

Figure II-9: Scanning electron photomicrographs of normal endothelium of a right middle cerebral artery from an animal injected with mock cerebrospinal fluid (top left, EBS). In contrast with this normal smooth endothelial surface, convolution and distortion of endothelium of a right middle cerebral artery is seen after injection of oxyhemoglobin (bottom left, OXYHB) or supernatant fluid (top right, SUPER). There is fibrin and debri adherent to endothelium after exposure to supernatant fluid. There are mild changes in endothelium in a middle cerebral artery from an animal injected with bilirubin (bottom right, BILI). All micrographs taken at 15 kV. Scale bars are present below each photomicrograph.

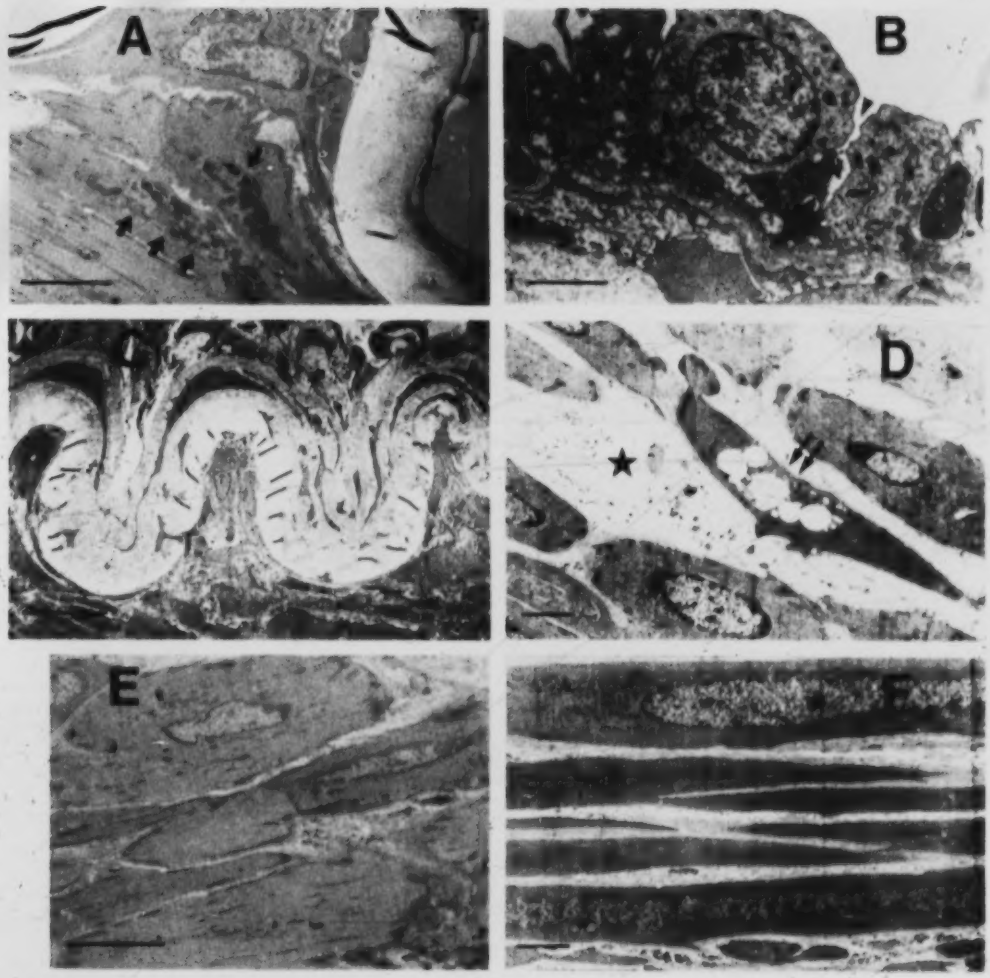

Figure II-10: Transmission electron photomicrographs of right middle cerebral arteries from animals injected with supernatant fluid (A and B), oxyhemoglobin (C and D), bilirubin (E), and mock cerebrospinal fluid (F). After injection of supernatant fluid (A), there is convolution of the internal elastic lamina, and contraction and dense body formation within medial smooth muscle (arrows). There is loss of tight junctions between endothelial cells (arrowheads, B). Similar changes are seen after injection of oxyhemoglobin (C and D) with pleating of internal elastic lamina, endothelial cell vacuolation, and smooth muscle contraction and vacuolation (double arrows). Star in D indicates a necrotic smooth muscle cell. After injection of bilirubin (E), there is some smooth muscle contraction but the cells are not damaged. F demonstrates normal medial smooth muscle in a middle cerebral artery from a monkey injected with mock cerebrospinal fluid. All photomicrographs taken at 75 kV. Scale bars on each photomicrograph are as follows: A, 5 μm; B, 2 μm; C, 10 μm; D, 2 μm; E, 5 μm; F, 2 μm.

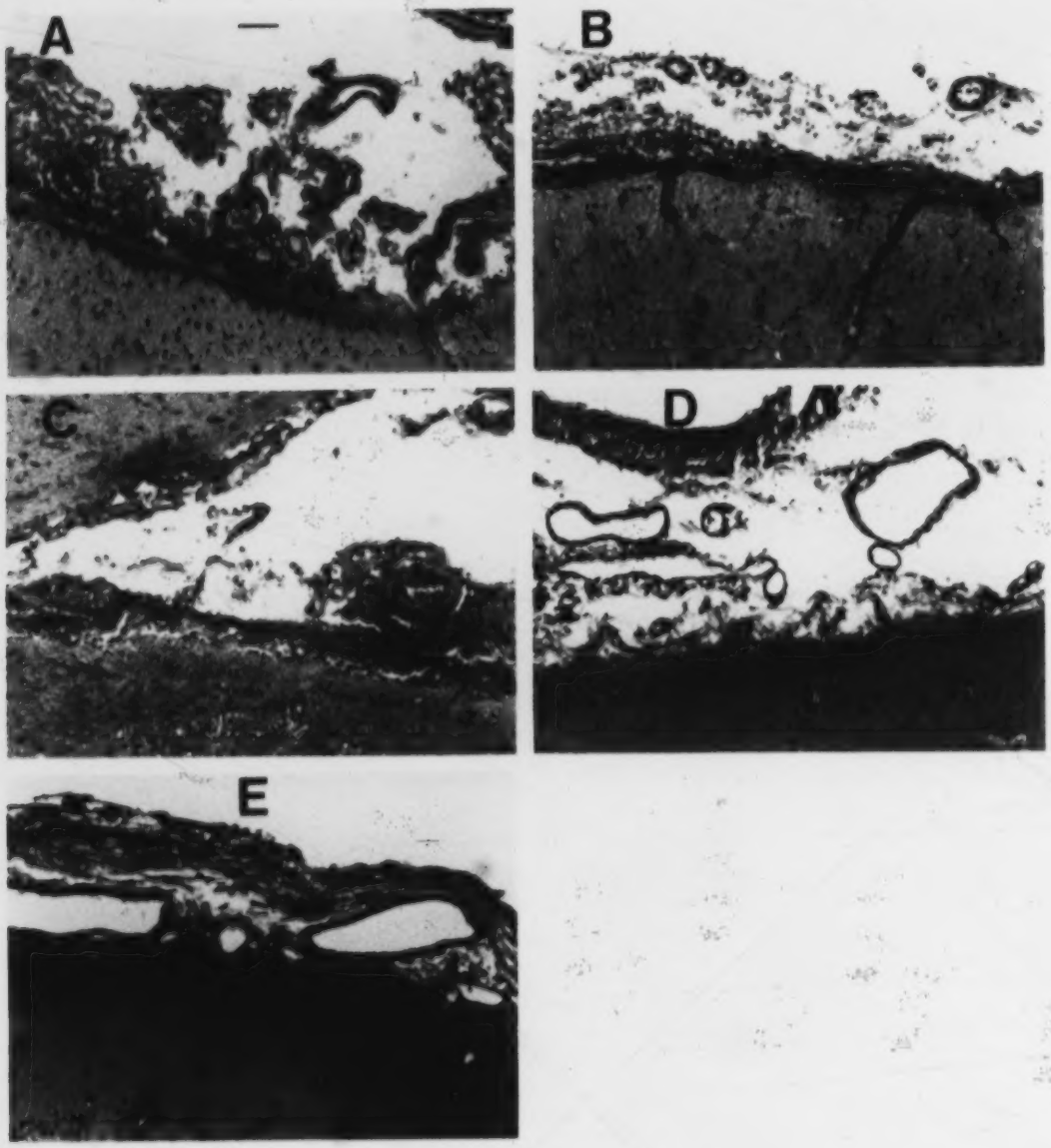

Figure II-11: Photomicrographs of brain and subarachnoid space of right sylvian fissures from monkeys in each group. There is moderate subacute inflammation consisting of macrophages, polymorphonuclear leucocytes, and fibrous tissue, in the subarachnoid space after injection of oxyhemoglobin (A) and methemoglobin (B). Injections of supernatant fluid (C) and bilirubin (D) produce less inflammation while the subarachnoid space of the animal injected with mock cerebrospinal fluid (E) is normal. Hematoxylin and Eosin. Scale bar in A (= 250 μm) applies to all photomicrographs in the illustration.

Figure II-12: Whole mount section of cerebrum of Monkey 31 (oxyhemoglobin group) showing a right middle cerebral artery-territory cerebral infarction (arrows) associated with mass effect and midline shift.

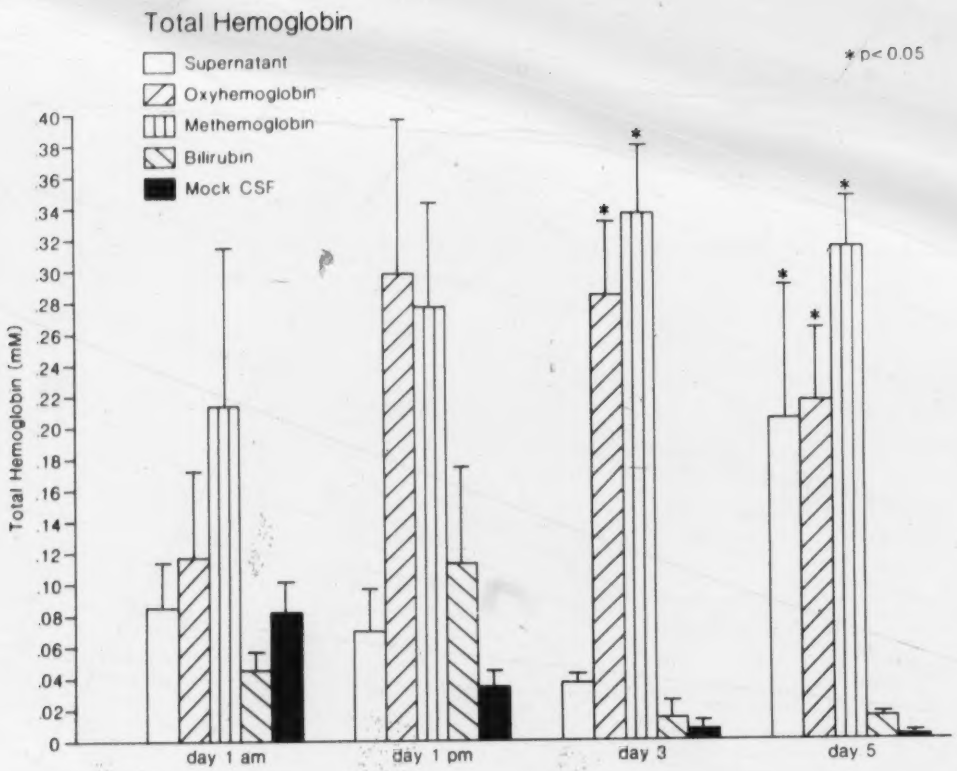

Figure II-13: Bar graph showing total concentration of hemoglobin in aspirated cerebrospinal fluid (CSF) of each group, by day. Bars represent means ± standard error (n = 8 for each bar). There is significant elevation of total hemoglobin on days 3 and 5 in groups receiving oxyhemoglobin and supernatant fluid and on day 5 in animals injected with supernatant fluid ($p < 0.05$, analysis of variance followed by Scheffe's multiple comparison procedure at each time period).

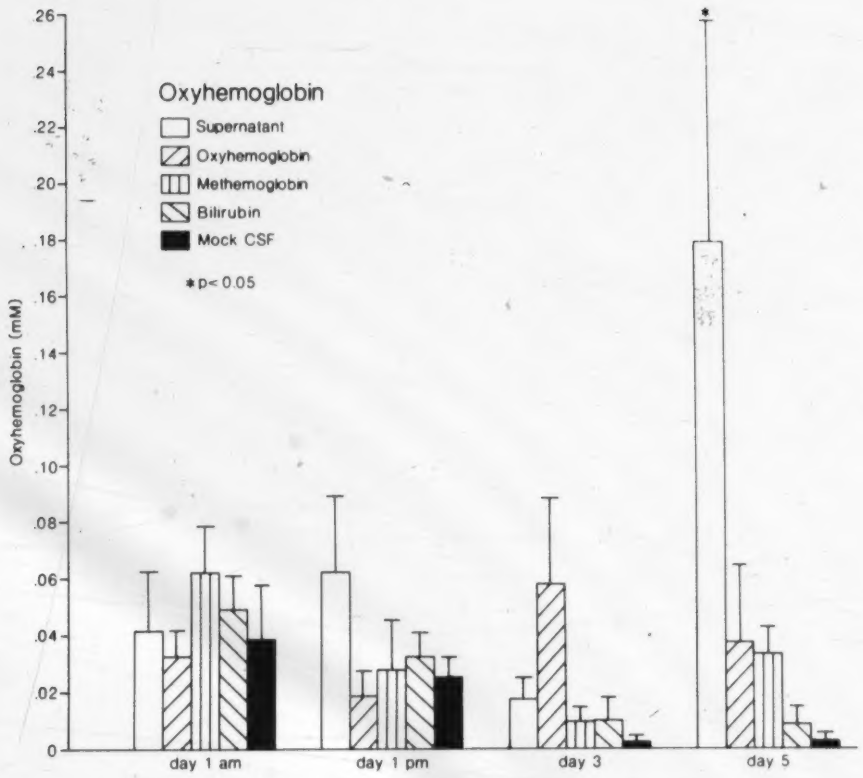

Figure II-14: Bar graph showing concentration of oxyhemoglobin in aspirated cerebrospinal fluid (CSF) of each group, by day. Bars represent means ± standard error (n = 8 for each bar). There is significant elevation in concentration of oxyhemoglobin on day 5 in the supernatant fluid group ($p < 0.05$, analysis of variance followed by Scheffe's multiple comparison procedure).

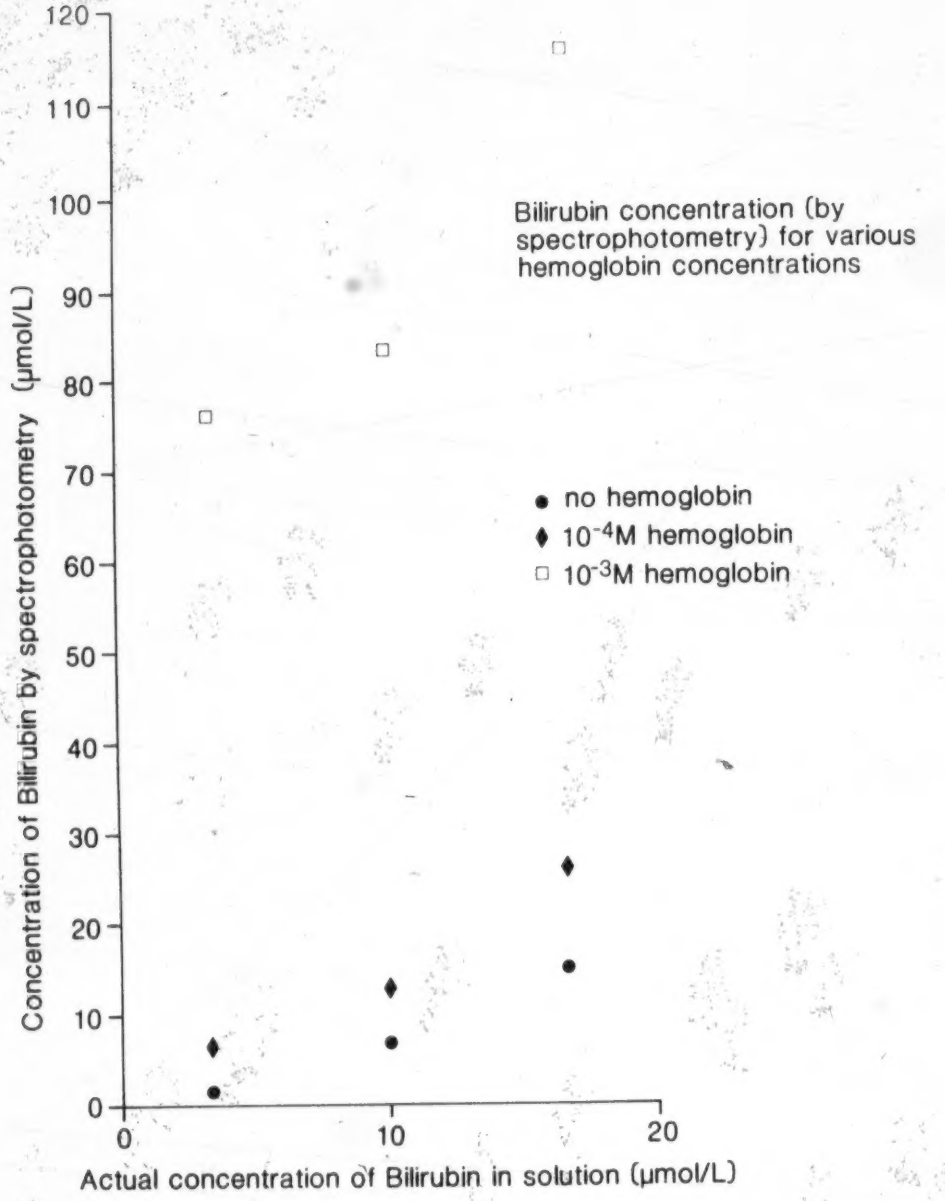

Figure II-15: Graph showing increasing concentrations of hemoglobin interfere with detection of bilirubin by Jendrassik-Grof analysis [18] (y axis) performed by a spectrophotometric method on a Parallel analyzer (American Monitor Corp., Indianapolis, Indiana).

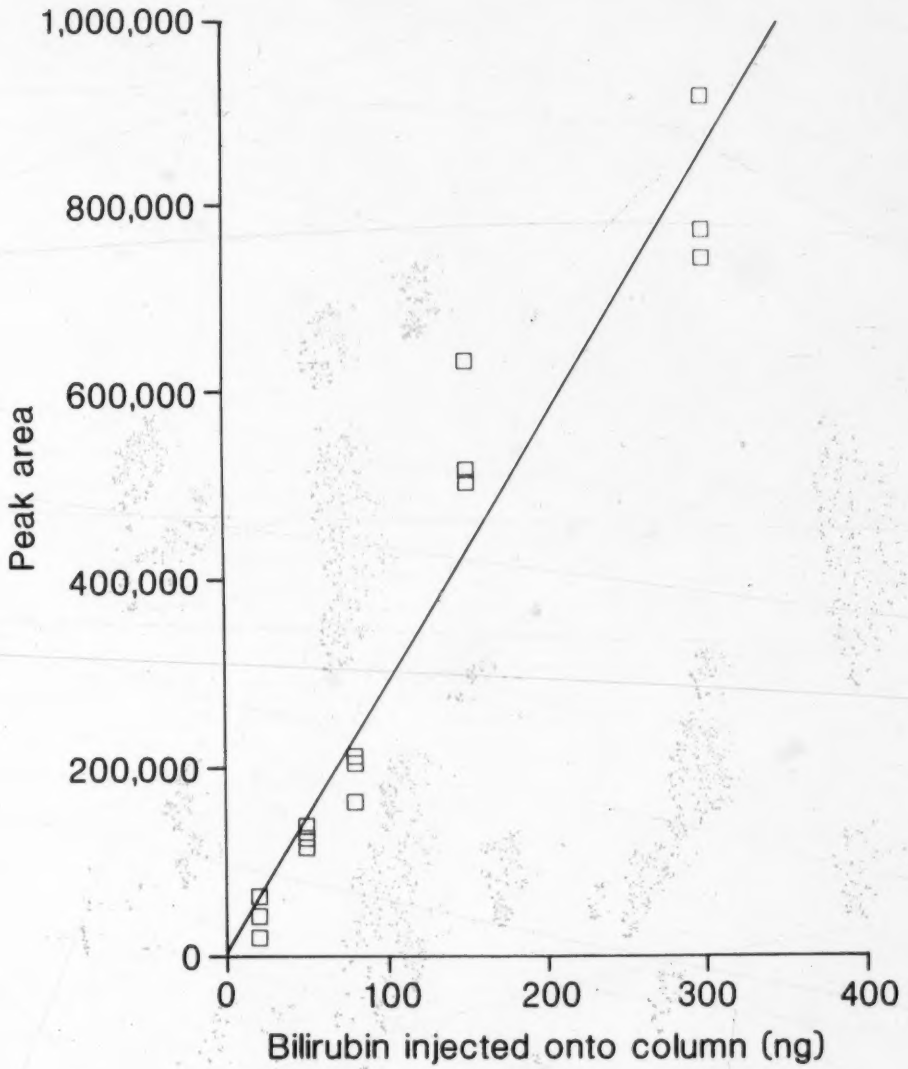

Figure II-16: Standard line for high pressure liquid chromatography analysis of bilirubin standards showing correlation of peak area with amount of bilirubin injected ($r = 0.966$, $p < 0.05$ by linear regression analysis).

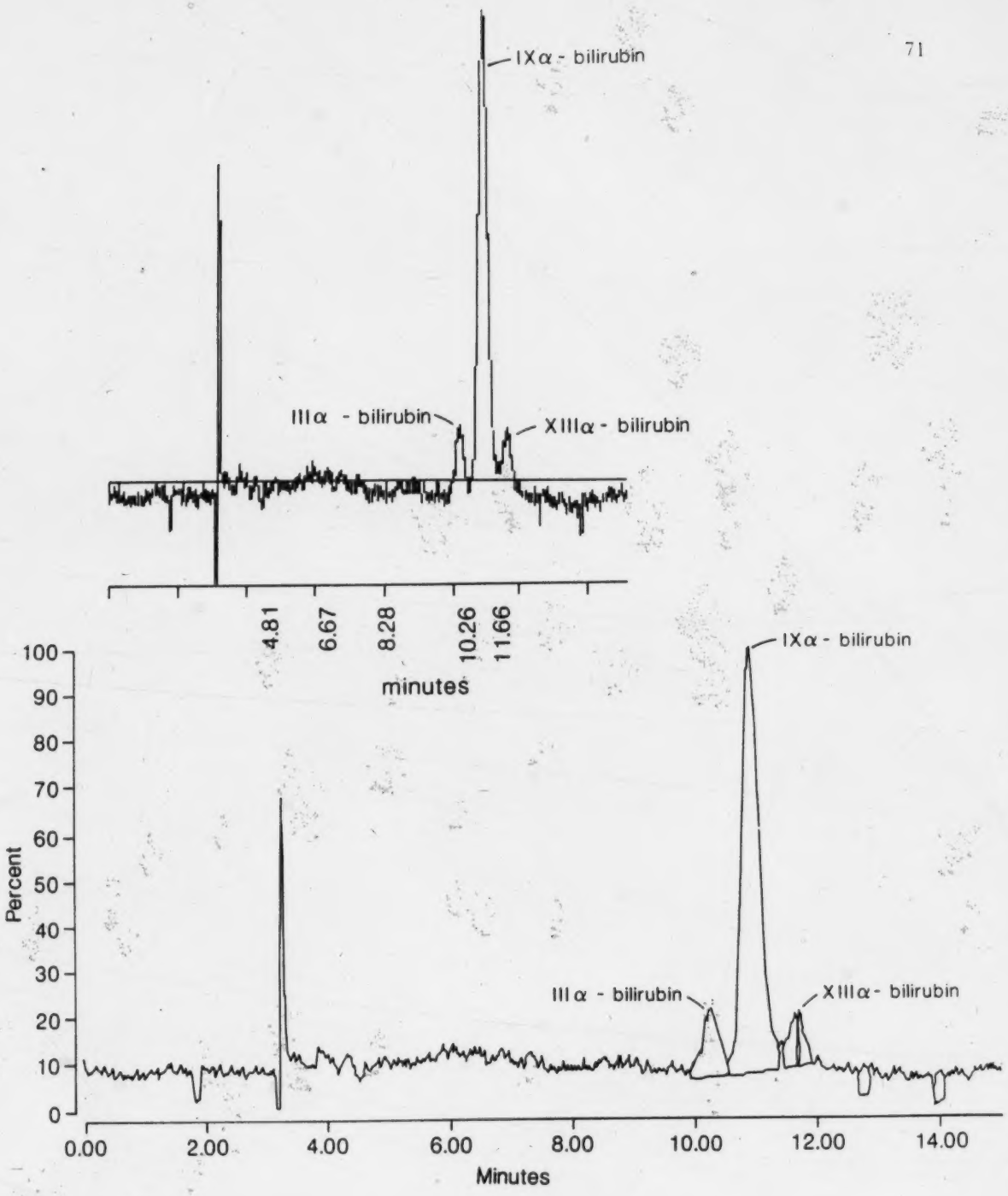

Figure II-17: Representative high pressure liquid chromatogram (top) and scaled plot (bottom) obtained after injection of an 80 ng bilirubin standard (see text for details of method). The 2 small peaks eluting before and after the main IXα-bilirubin peak are due to the IIIα- and XIIIα- isomers of bilirubin.

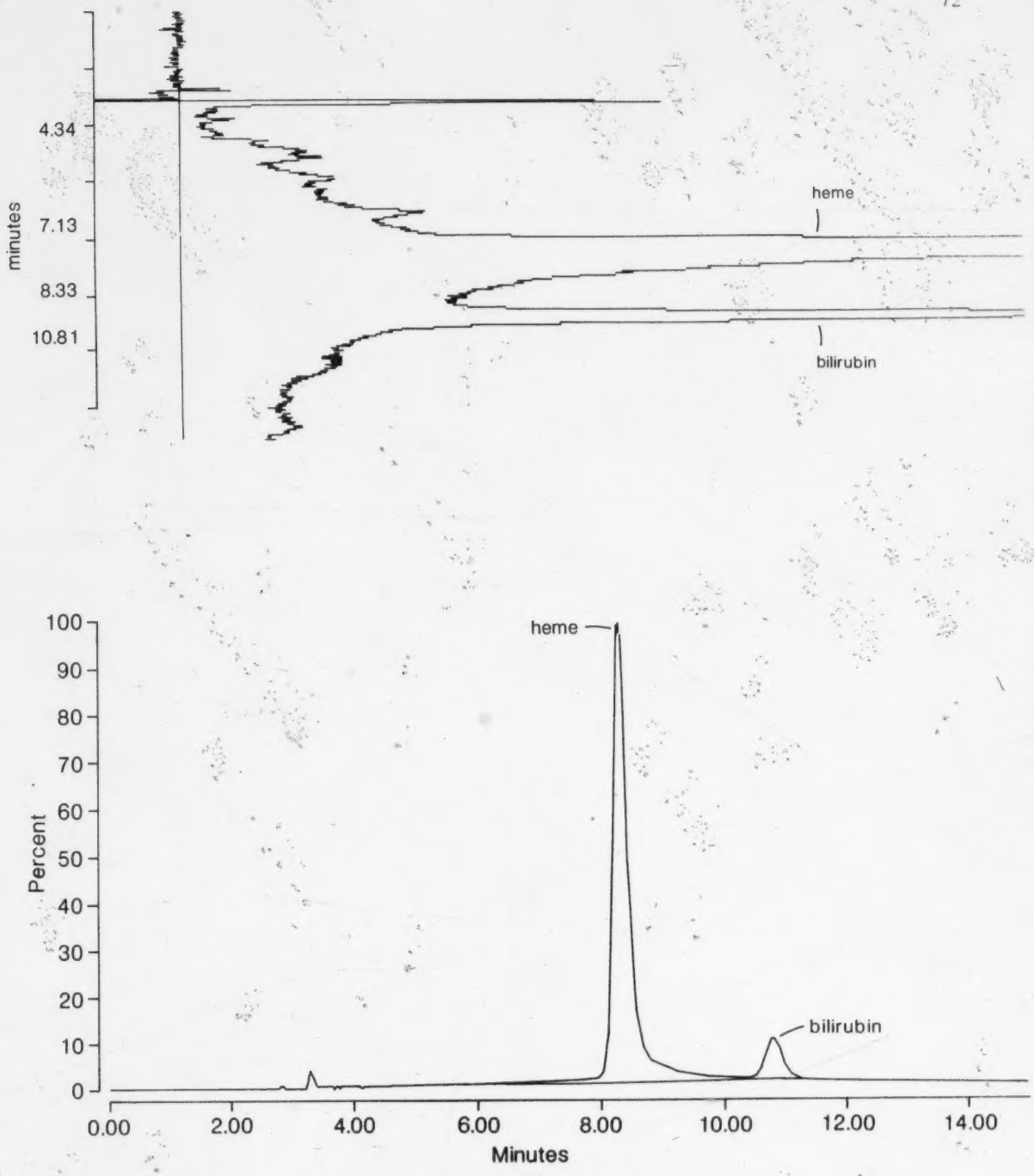

Figure II-18: Representative high pressure liquid chromatogram (top) and scaled plot (bottom) obtained after injection of a sample of cerebrospinal fluid from an animal injected with methemoglobin. Heme elutes at approximately 8 minutes and is separated from bilirubin which appears after 10 minutes. See text for details of methods.

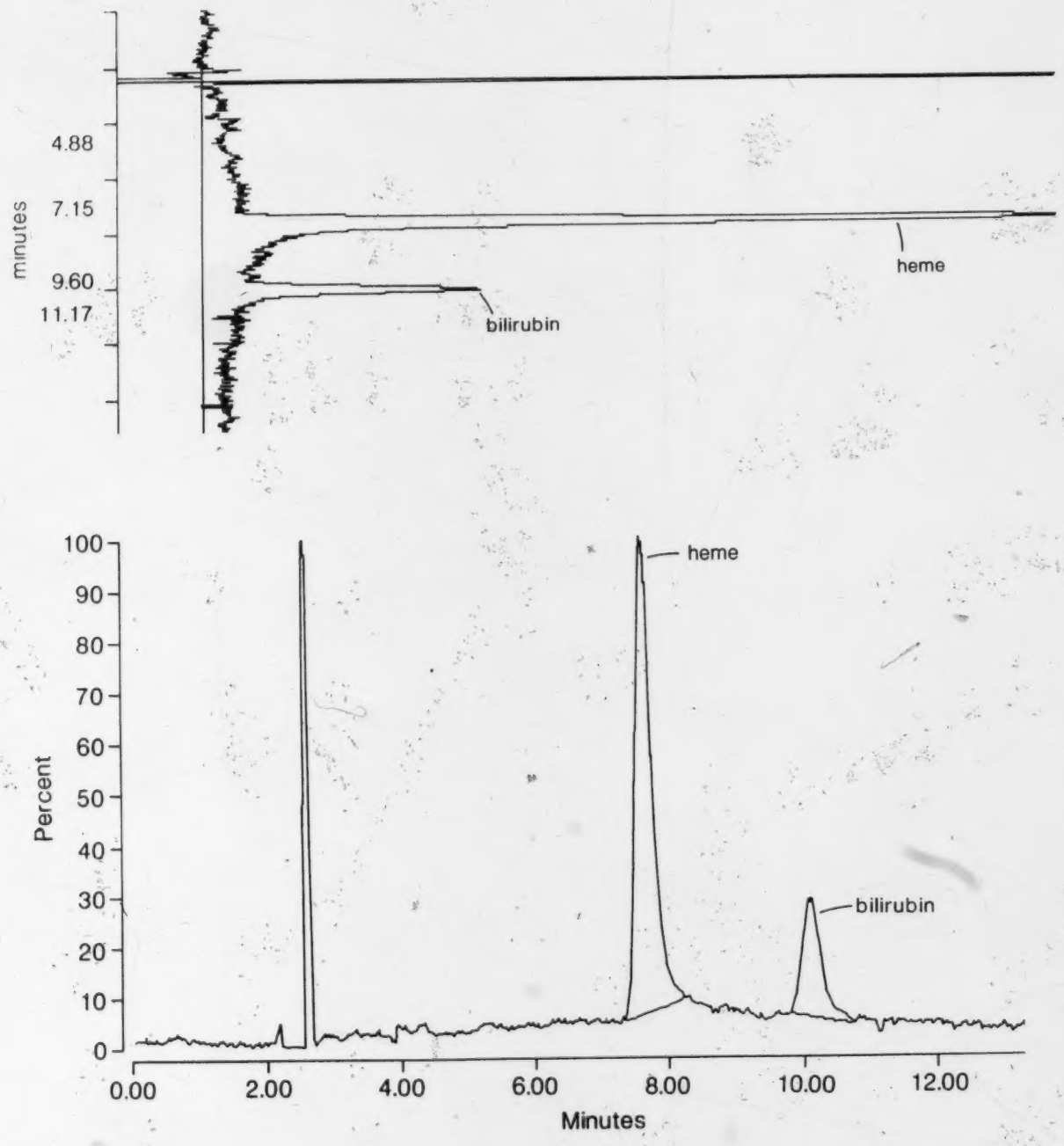

Figure II-19: Representative high pressure liquid chromatogram (top) and scaled plot (bottom) obtained after injection of a sample of cerebrospinal fluid from an animal injected with supernatant fluid. Heme elutes at approximately 8 minutes and is separated from bilirubin which appears after 10 minutes. See text for details of methods.

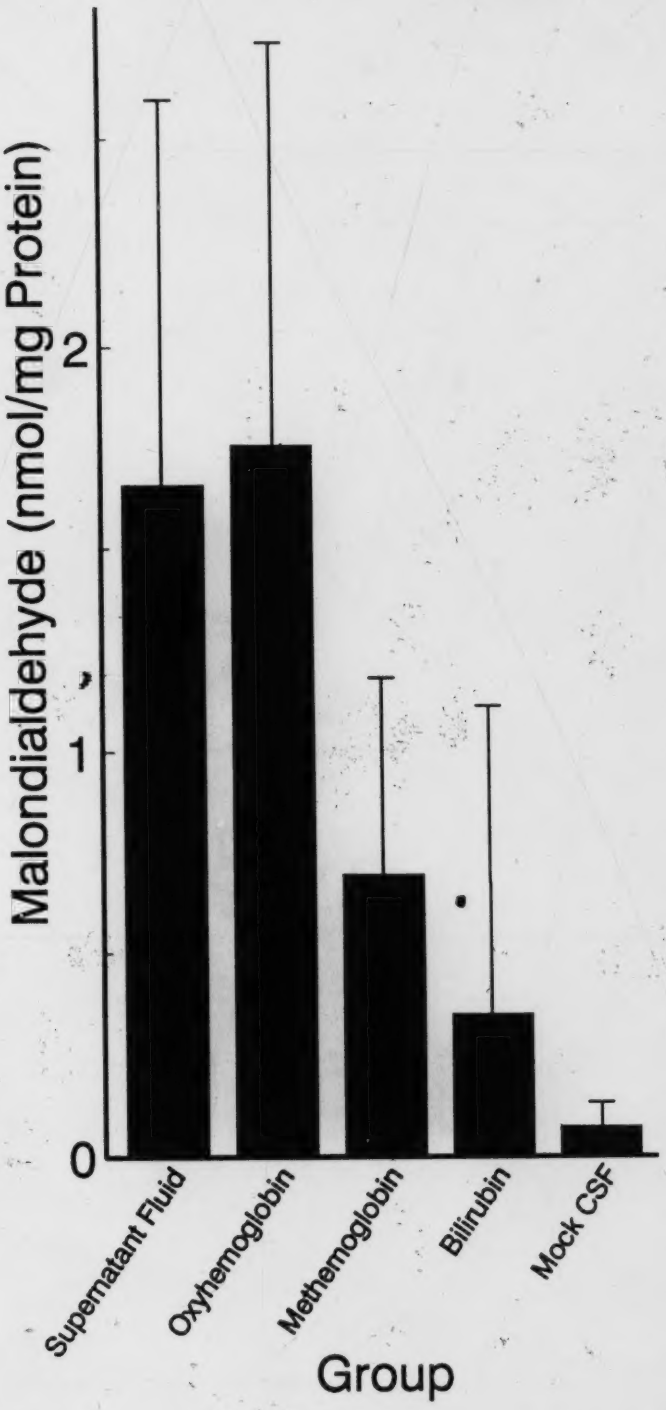

Figure II-20: Bar graph of mean concentration of malondialdehyde (bars represent nmol malondialdehyde/mg protein ± standard deviation) in cerebrospinal fluid (CSF) aspirated from each group. 24 samples were analyzed in each group. There were no significant differences between groups (Kruskal-Wallis test).

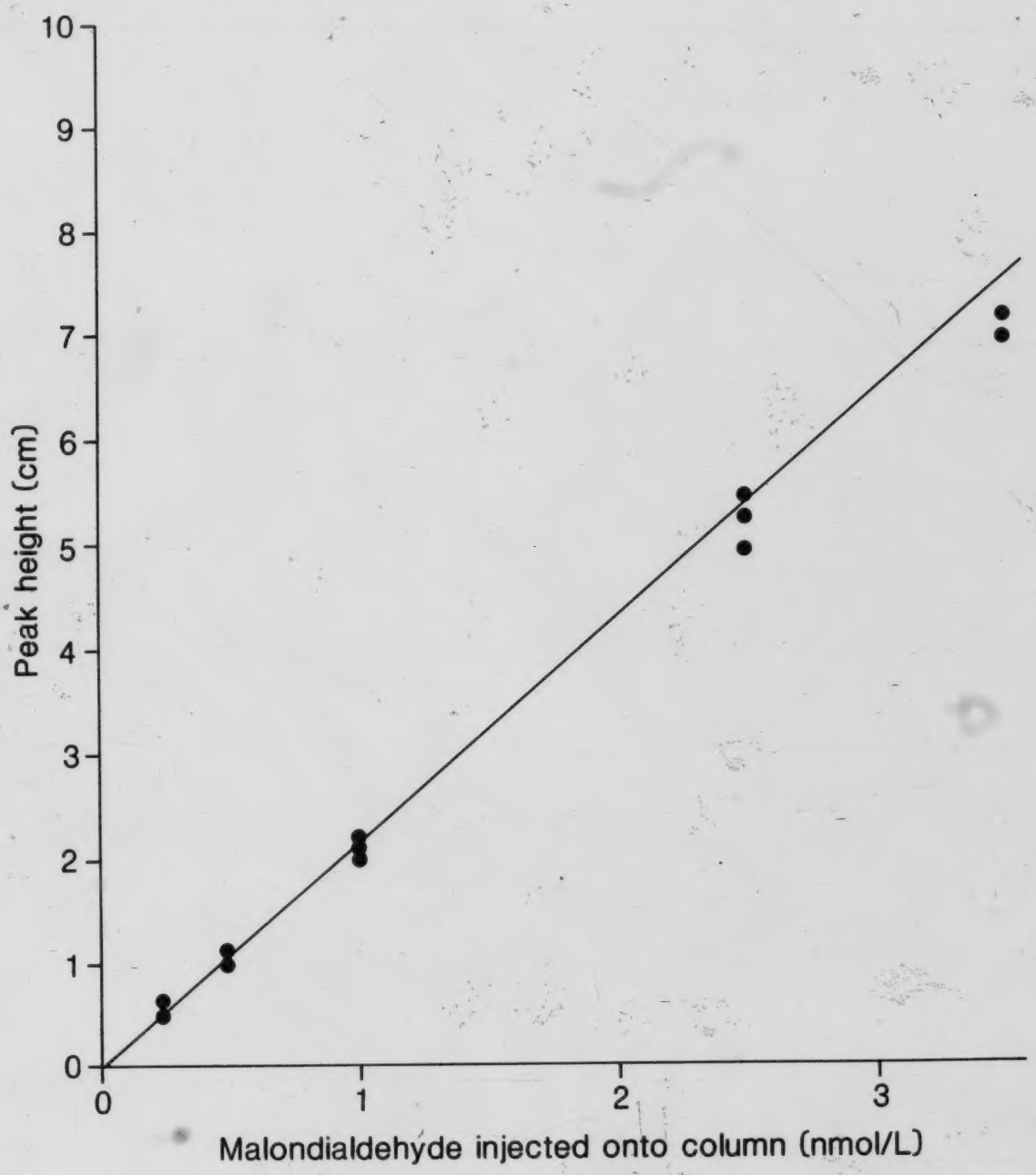

Figure II-21: Standard line showing correlation of peak height on high pressure liquid chromatograms with concentration of malondialdehyde injected onto the column ($r = 0.993$, $p < 0.0001$, by linear regression analysis).

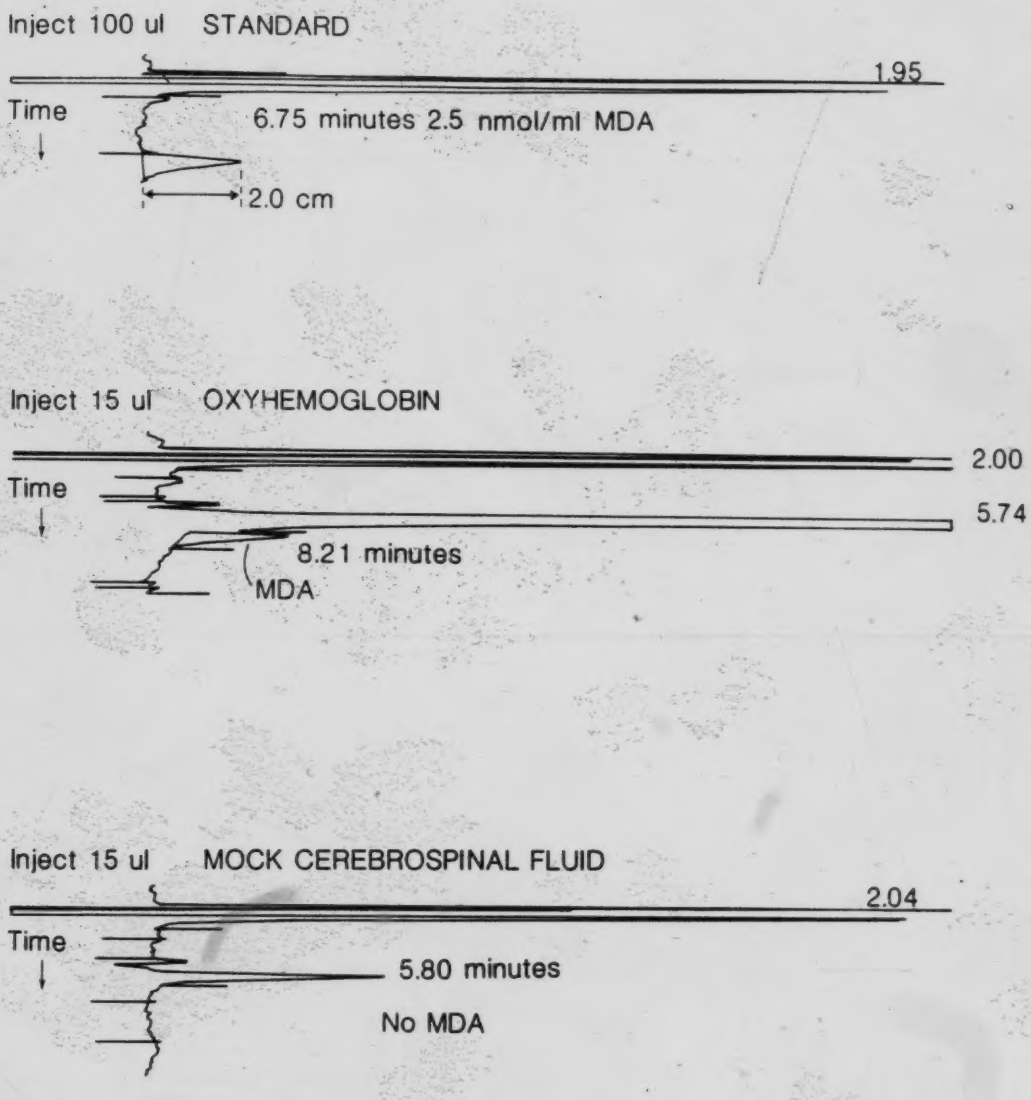

Figure II-22: Representative high pressure liquid chromatograms for analysis of malondialdehyde (MDA). The top chromatogram shows malondialdehyde eluting 6.75 minutes after injection of an MDA standard. Center chromatogram shows MDA peak 8.21 minutes after injection of a cerebrospinal fluid sample from a monkey injected with oxyhemoglobin. Bottom chromatogram shows absence of an MDA peak after injection of cerebrospinal fluid from a monkey in the mock cerebrospinal fluid group.

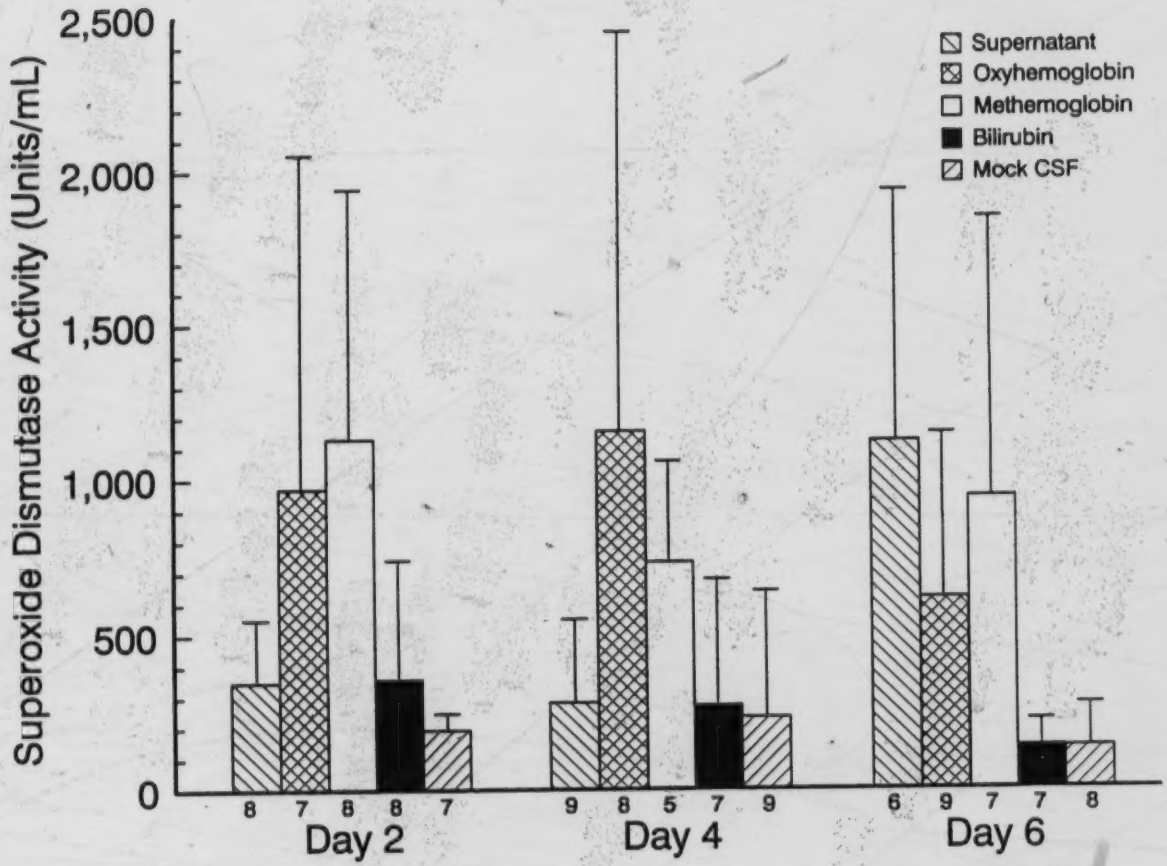

Figure II-23: Mean activity of superoxide dismutase (bars represent Units of superoxide dismutase activity/ml ± standard deviation) in cerebrospinal fluid (CSF) aspirated from each group on days 2, 4, and 6. Number of samples analyzed is shown below each bar. Analysis of variance failed to reveal significant variance at any time period, although activities were generally higher after injection of supernatant fluid, oxyhemoglobin, and methemoglobin.

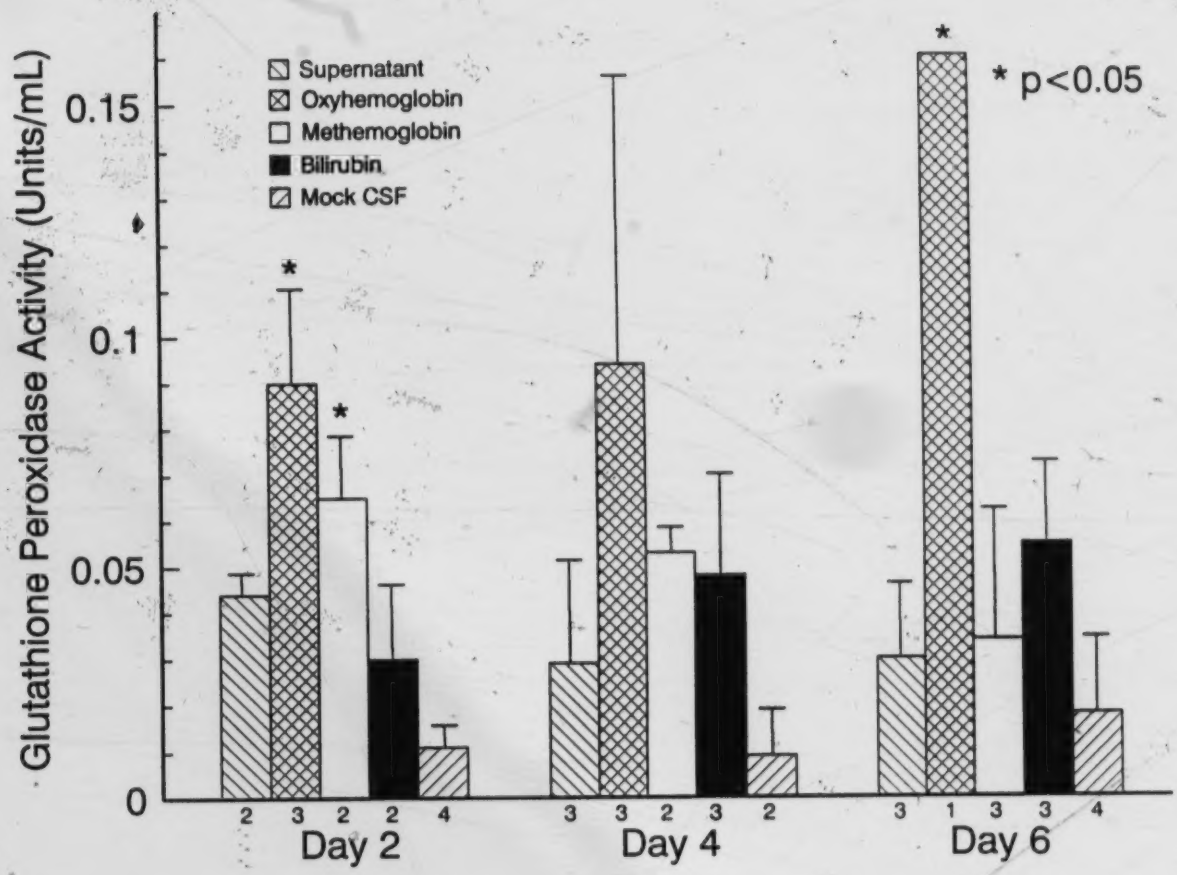

Figure II-24: Mean activity of glutathione peroxidase (bars represent glutathione peroxidase activity in Units/ml ± standard deviation) in cerebrospinal fluid aspirated from each group on days 2, 4, and 6. Number of samples analyzed is shown below each bar. Activity is significantly greater than other values ($p < 0.05$, analysis of variance followed by Scheffe's multiple comparison procedure) in oxyhemoglobin group on day 6 and in oxyhemoglobin and methemoglobin groups on day 2 ($p < 0.05$, analysis of variance followed by Scheffe's multiple comparison procedure).

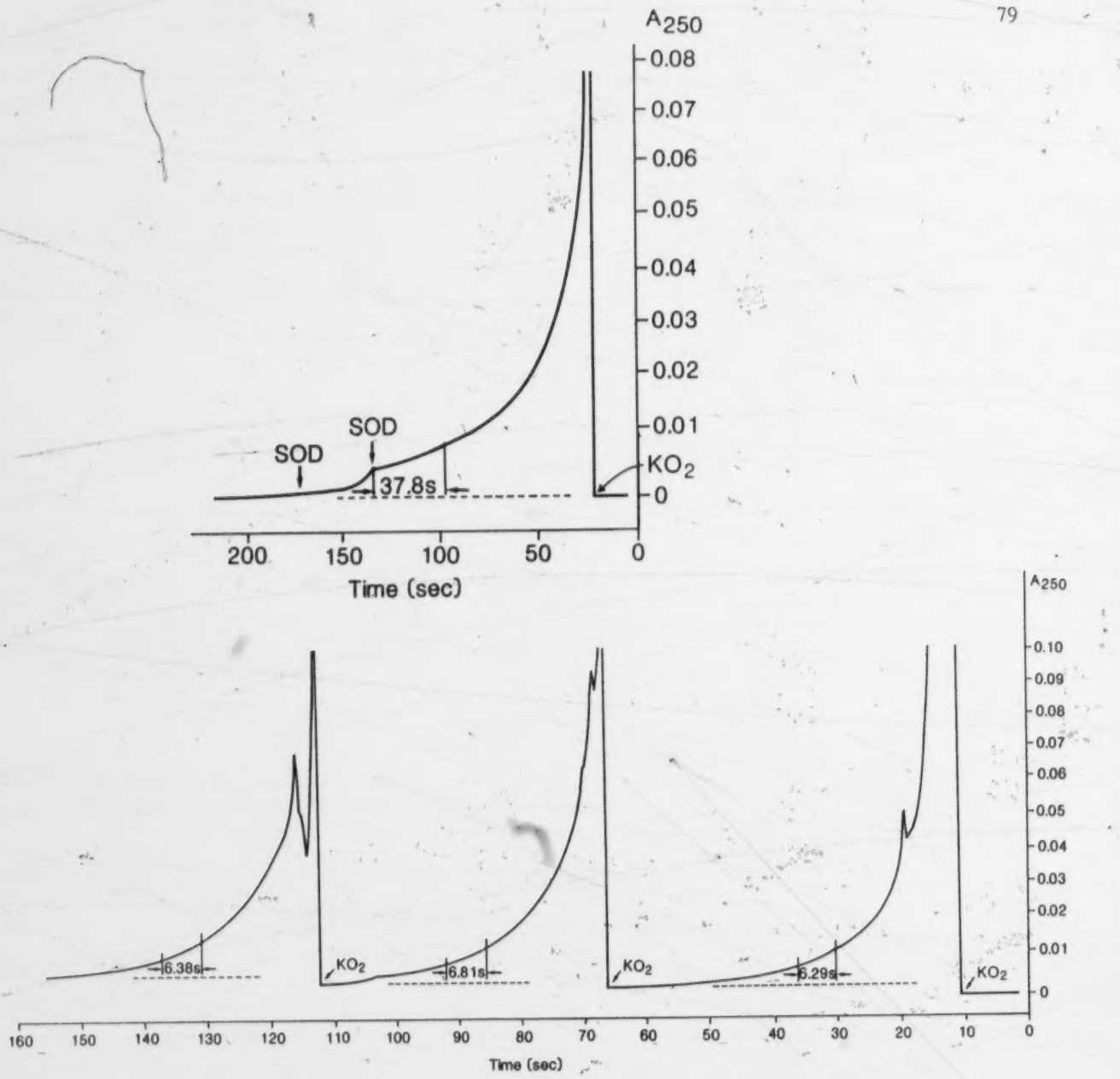

Figure II-25: Top: Representative plot of absorbance versus time for superoxide dismutase (SOD) assay of a sample containing no SOD activity. Potassium superoxide (KO_2) is added on the right and absorbance, due to superoxide, is followed at 250 nm. Half-life for loss of superoxide is calculated and from this a rate constant, which is related to enzyme activity, can be derived. Bottom: Similar plot assaying cerebrospinal fluid aspirated from an animal injected with oxyhemoglobin. For each sample, addition of KO_2 is performed 3 times. The shorter half-life indicates activity of SOD is present.

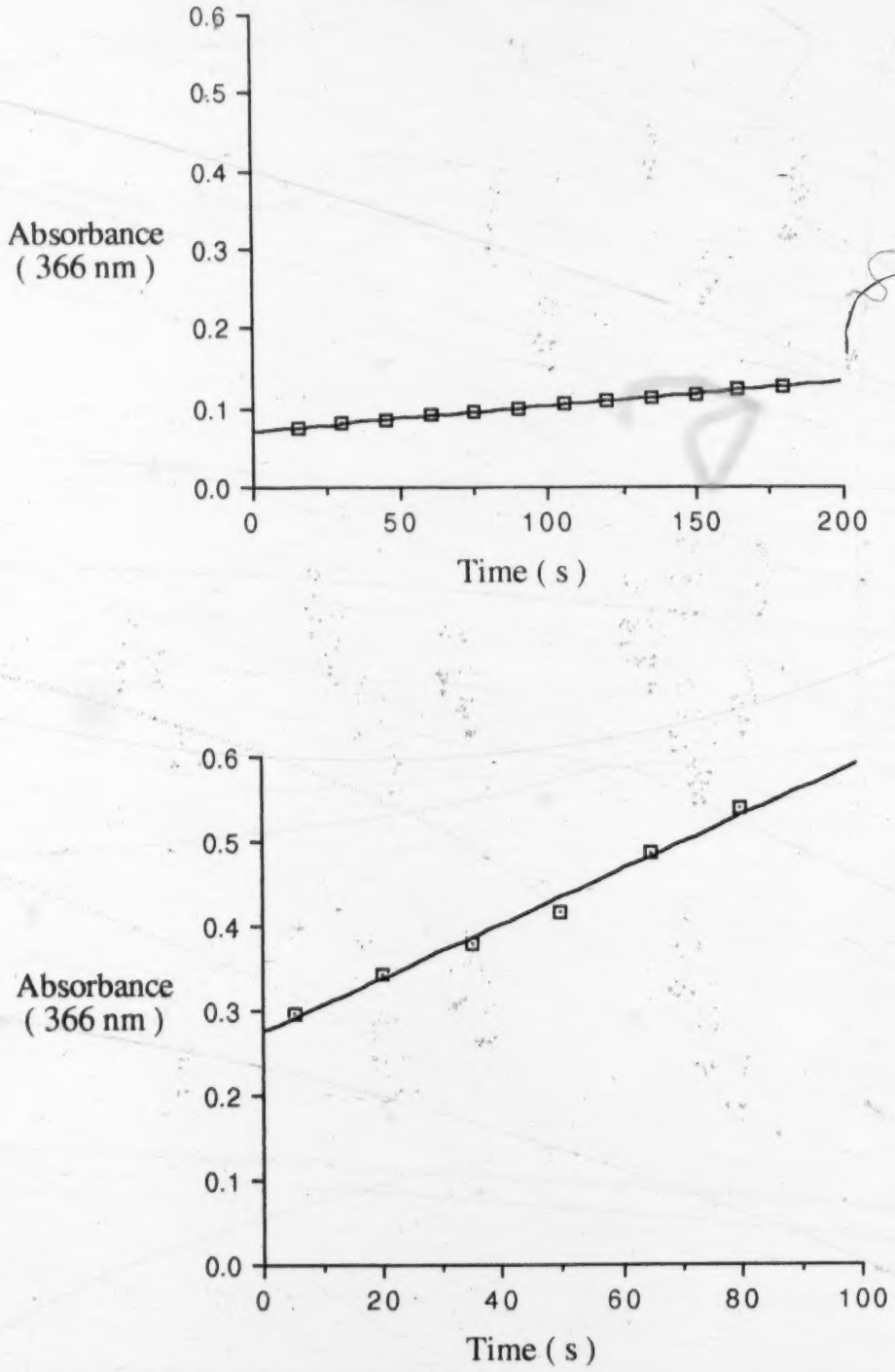

Figure II-26: Absorbance plots for glutathione peroxidase assay. Top: Plot of change in absorbance at 366 nm with time for a sample of water. Bottom: Plot of change in absorbance versus time for sample of cerebrospinal fluid aspirated on day 2 from an animal injected with supernatant fluid. Rate of change is greater than that occurring in the top control plot due to presence of glutathione peroxidase activity.

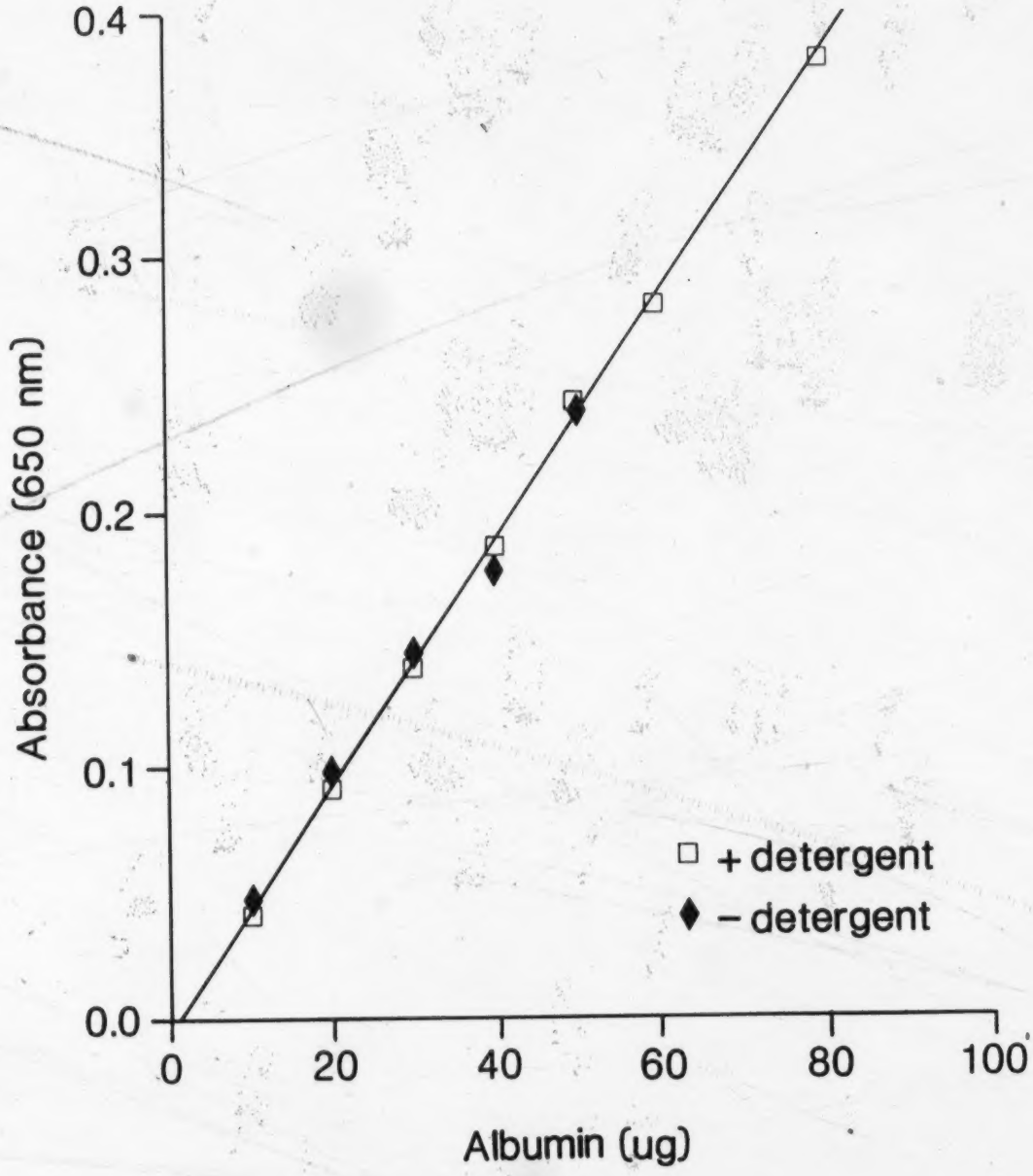

Figure II-27: Standard line for protein determinations, with and without detergent, by Lowry assay [31] showing correlation between amount of bovine serum albumin and absorbance at 650 nm ($r = 0.999$, $p < 0.00001$, by linear regression analysis). Detergents did not interfere with protein determination by this assay.

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CHAPTER THREE [a]
EFFECT OF INTRATHECAL SUPEROXIDE DISMUTASE AND CATALASE ON OXYHEMOGLOBIN-INDUCED VASOSPASM IN MONKEYS

SUMMARY

A gel consisting of agarose and oxyhemoglobin (OxyHb) was developed so that, when placed in the subarachnoid space, OxyHb would be slowly released, simulating lysis of erythrocytes after subarachnoid hemorrhage (SAH). The system was used to investigate importance of free radical-mediated reactions in genesis of OxyHb-induced vasospasm (VSP) in monkeys. Seventeen monkeys were randomly assigned to have subarachnoid placement, on day 0, of one of the following: 1. agarose gel alone (n = 2), 2. agarose plus OxyHb (n = 3), 3. agarose plus OxyHb plus intrathecal administration of superoxide dismutase (SOD) and catalase (n = 6), and 4. agarose plus OxyHb plus intrathecal administration of placebo (n = 6). Vasospasm was assessed by comparison of angiograms performed on day 0 and 7 days after placement of subarachnoid compounds and by electron microscopy. Oxyhemoglobin alone caused significant reduction in middle cerebral artery (MCA) diameter ($p < 0.05$) which was associated with ultrastructural damage to smooth muscle. Treatment with SOD plus catalase or with placebo attenuated MCA VSP, although significant narrowing persisted in both groups ($p < 0.05$). Analysis of variance showed no difference in degree of VSP between groups exposed to subarachnoid OxyHb. Cerebrospinal fluid (CSF) aspirated from the cisterna magna on day 7 contained elevated activity of SOD in animals which received treatment. Malondialdehyde was undetectable in CSF after subarachnoid agarose placement although it was present in similar amounts in all groups which had subarachnoid placement of OxyHb. Since intrathecal SOD and catalase failed to protect against OxyHb-induced VSP, free-radical mediated mechanisms may not be important in its genesis. However, as only one combination of doses of SOD and catalase was administered, it may be that other dosage schedules might be efficacious.

INTRODUCTION

There is substantial clinical and experimental evidence that OxyHb released from hemolyzing subarachnoid erythrocytes mediates VSP after aneurysmal SAH.[25,40,48] The mechanism of action of OxyHb is unclear and several possible mechanisms of action of OxyHb within arterial wall have been reported.[1,7,22,23,26,28,31,36,40,42,43] Oxyhemoglobin may cause VSP through a free radical-mediated mechanism.[1,36] Oxyhemoglobin penetrates cerebral arterial walls after SAH.[24] Therefore, OxyHb, or ferrous iron released from it, might cause VSP by generating free radicals directly on arterial smooth muscle. Evidence that free radicals mediate disease is usually obtained by experiments which detect free radical reaction products such as lipid peroxides, and associate them with disease development.[16] Alternatively, many laboratories have shown that inhibitors of free radical reactions may prevent the disease or damage.

Vasospasm in humans [1,21,33,36,42,47] and experimental animals [19,34] is associated with lipid peroxidation. There are, however, limitations to methods which detect lipid peroxides since VSP damages arterial walls and tissue damage can lead secondarily to lipid peroxidation, regardless of the mechanism of tissue damage.[13,14] Therefore, better evidence that free radicals mediate VSP would demonstrate that VSP develops before lipid peroxides appear.

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Alternatively, inhibition of VSP in monkeys by a specific inhibitor of iron-dependent lipid peroxidation, U74006F, is evidence for a role of free radicals in VSP.[18,41] Experiments *in vitro* do not consistently show whether inhibition of free radical reactions can prevent cerebral artery contraction by OxyHb.[11,17,31,40,49] We are unaware of any experiments using animal models *in vivo* where OxyHb is the spasmogen and where free radical scavengers are used to treat such spasm.

Vasospasm was produced in monkeys using a gel composed of agarose and OxyHb. The rationale for using agarose gel was to permit slow release of high concentrations of pure OxyHb adjacent to cerebral arteries without the potentially compounding influences of other clot constituents or the necessity of repeated intrathecal injections. Contribution of free radical reactions to OxyHb-induced VSP was assessed by treating spasm with free radical scavenging enzymes, SOD and catalase. This combination of scavengers was chosen for several reasons. Superoxide dismutase alone could fail to protect against VSP induced by OxyHb.[11,31,40,49] Superoxide dismutase catalyzes a reaction that produces hydrogen peroxide which, in the presence of ferrous iron and certain ferrous hemoproteins, including OxyHb, could produce hydroxyl free radical by an iron-catalyzed Haber-Weiss reaction. Cerebrospinal fluid contains litte catalase or glutathione peroxidase,[12] enzymes which could remove hydrogen peroxide, inhibiting production of hydroxyl free radical. Hydrogen peroxide also can inactivate SOD.[15] Hydrogen peroxide also degrades heme, releasing iron.[14] Presence of catalase would remove hydrogen peroxide, prevent inactivation of SOD, decrease the amount of free iron in the subarachnoid space,[45] and possibly reduce levels of hydroxyl free radicals, the most potent of the class of oxygen-derived free radicals. Enzymes were given intrathecally as they would not cross an intact blood-arterial wall barrier.

MATERIALS AND METHODS

Protocol

Seventeen male monkeys (Macaca fascicularis) weighing between 2.0 and 2.6 kg were divided by restricted randomization into 4 groups (Fig. III-1). On day 0, animals underwent baseline cerebral angiography, right craniectomy, and arachnoid dissection. Each animal had subarachnoid placement of one of the following next to exposed cerebral arteries of the right side of the circle of Willis: agarose gel in group 1 ($n = 2$) or agarose plus OxyHb in groups 2, 3, and 4 ($n = 3$, 6, and 6, respectively). In groups 3 and 4, intrathecal catheters were placed along the right sylvian fissure and connected to osmotic pumps (Alzet Osmotic pump model 2ML1, Alza Corporation, Palo Alto, California) anchored in the subcutaneous tissue over the dorsal thorax. Group 3 received 5×10^5 U catalase and 5×10^5 U human recombinant copper/zinc SOD (kindly donated by Chiron Corporation, Emeryville, California, lot number MEA086) administered continuously over 7 days by osmotic pump. Group 4 had intrathecal catheters and subcutaneous pumps filled with placebo consisting of inactivated SOD plus additional protein as bovine serum albumin to account for protein in catalase. Vasospasm was assessed by comparing baseline angiography with angiography done 7 days later, and by transmission electron microscopy. Concentrations of malondialdehyde (MDA) and protein and activities of SOD and catalase were measured in CSF aspirated from cisterna magna on day 7. Bilirubin content was measured in 2 gels from monkeys exposed to OxyHb-containing gel but which were not treated with placebo or SOD plus catalase.

Baseline angiography and operative procedures were blinded until completion of arachnoid dissection when the operator was given a box containing materials for implantation, according to randomization. Interpretation of angiograms, biochemical analyses, and data analysis were blinded.

Procedures involving animals were approved by the Animal Ethics Review Committee of University of Alberta and were carried out in accord with recommendations of the Canadian Council on Animal Care.

Subarachnoid Gel Preparation

Oxyhemoglobin was prepared from purified crystalline human hemoglobin (Sigma Chemical Co., St. Louis, Missouri) by the method of Bauer and Pacyna.[3] Ten g hemoglobin was suspended in deionized water and stirred for 3 hours at 4°C. The mixture was centrifuged at 450 x g for 10 minutes and dialyzed against 8 L deionized water at 4°C for 36 hours. Sodium dithionite (Sigma Chemical Co., St. Louis, Missouri), 62.5 mg, was added to 12.5 ml N_2-purged deionized water and this solution added to 24 ml hemoglobin solution. The solution was immediately applied to a 1.5 X 50 cm column of mixed-bed ion-exchange resin (AG 501-X8 (D), BioRad, Richmond, California) and eluted with deionized water at 150 ml/h. Product was concentrated by ultrafiltration (Amicon PM 30 membranes) to a final concentration of 3 mM in a solution with pH, osmolarity, electrolyte, and glucose content similar to CSF (Elliott's solution B [8]). Spectrophotometry showed the solution contained 78% OxyHb.[4] For production of gels, low temperature gelling agarose (type VII, Sigma Chemical Co., St. Louis, Missouri) was prepared at 1.85% in deionized water and allowed to cool to less than 35°C before adding OxyHb solution (1.8 ml of 3 mM solution containing 350 mg protein) to 1.2 ml agarose. After mixing, the solution was drawn into a 3 ml syringe and allowed to set at 4°C for 2 hours prior to implantation. Thus, each animal had 350 mg OxyHb placed in the subarachnoid space, an amount contained in 3 ml monkey blood. Previous experiments with a cynomolgus monkey model of VSP showed 3 to 5 ml clotted arterial blood caused VSP when placed in the subarachnoid space.[10] Two control animals had subarachnoid placement of 3 ml pure agarose without addition of OxyHb.

Preparation of Enzymes

One g of crystalline bovine liver catalase (Boehringer Mannheim Canada, Ltd., Laval, Quebec) was washed extensively with ice-cold deionized water and dissolved in Elliott's solution B at a final concentration of 240 μM and activity of 3.4×10^6 U/ml. Two g of human recombinant SOD were reconstituted in 28 ml Elliott's solution B and dialyzed against 2 changes of 4 L of this buffer over 24 hours. For placebo, inactivated SOD and albumin were used. A solution of SOD, 70 mg/ml, was inactivated over 1 hour at 4°C by addition of H_2O_2 at a final concentration of 2%.[15] Inactivated enzyme was dialysed over 12 hours against 2 changes of 2 L Elliott's solution B at 4°C. Product was concentrated by ultrafiltration to the same concentration of SOD used in treated monkeys (70 mg/ml). Less than 5% of initial activity remained after inactivation. A 5% solution of bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri) in Elliott's solution B was heated to 80°C for 2 hours to remove endogenous SOD and catalase activity. The solution was diluted to contain albumin at 240 μM. Volumes of 440 μL were used in the placebo group to make up for additional protein contributed by catalase. All solutions were sterilized by filtration through 0.22 μm filters and stored for up to 2 weeks at -80°C. Freezing did not affect enzyme activity.

Osmotic pumps were prepared under sterile conditions prior to implantation. Each pump was filled with either 5×10^5 U SOD plus 5×10^5 U catalase (total volume 2 ml) or the same volume and protein concentrations of inactivated SOD and bovine serum albumin. Medical grade vinyl tubing (PE-60 tubing, Bolab, Inc., Lake Havasu City, Arizona) was connected to the flow moderator of the pump and the apparatus primed in 30 ml Elliott's solution B at 37°C for 4 hours prior to implantation.

Preliminary Experiments

An agarose gel containing 350 mg hemoglobin was incubated in Elliott's solution B *in vitro* at 37°C and the solution sampled daily to assess rate of release of hemoglobin from agarose. An osmotic pump was filled with SOD and catalase exactly as for implantation *in situ* except it was incubated in Elliott's solution B *in vitro* at 37°C for 7 days. A catheter connected to the pump was placed in a second tube containing 2 ml

Elliott's solution B which was changed every second day and assayed for activity of SOD and catalase. An osmotic pump and intrathecal catheter filled with SOD and catalase was implanted in a single cynomolgus monkey exactly as for the experimental protocol except that no subarachnoid gel was placed. A second intrathecal catheter was placed along the sylvian fissure adjacent to the first catheter. This catheter was connected to a subcutaneous CSF reservoir (Ommaya reservoir, American V. Mueller, Chicago, Illinois) to permit daily CSF sampling.

Surgical Procedures

Baseline physical assessment and cerebral angiography, right craniectomy, and arachnoid dissection were carried out as previously described with the following changes.[9,10] Monkey body temperature was maintained during all procedures by a water-filled heating pad (Aquamatic model K-20-C, American Hospital Supply Corporation, Valencia, California). All angiograms were performed through a 2 inch, 20 guage polyethylene catheter inserted under sterile conditions and magnification through an arteriotomy in the right axillary artery. Angiograms were obtained by injecting 10 ml iothalamate meglumine at 50 psi via the Cordis injector (Corids Corp., Miami, Florida).

Following arachnoid dissection, gel was placed next to exposed arteries. Dura was closed in water-tight fashion with a patch of superficial temporalis fascia sewn in place with 6-0 silk suture. In animals which had pumps placed, a subcutaneous pocket was created leading from the cranial incision to skin over the back. Pumps were advanced down the space until they lay in subcutaneous tissue over the back. The catheter was threaded through the dura to lie along the sylvian fissure. It was secured in place with suture and dental cement. Closure was as previously described.[9,10]

Biochemical Measurements

Malondialdehyde was measured by high pressure liquid chromatography.[5] A Waters series 3B liquid chromatograph and Waters spectrophotometer set at 267 nm were used. Chromatographic separations were performed on a Whatman chromasorb C1B 5 micron ODS column (4.6 x 150 mm) with a mobile phase of 14% (v/v) acetonitrile in 50 mM myristyltrimethylammonium bromide and 1 mM sodium phosphate (pH 6.4) run at 1.0 ml/min at room temperature. Superoxide dismutase activity was measured by direct assay with potassium superoxide as described by Marklund.[27] The method is given in detail in Chapter Two (page 43). One unit of activity was defined as the activity that disproportionates superoxide anion radical at a rate of 0.1 per second. Catalase activity was measured by direct substrate depletion assay. Samples were diluted in 0.05 M phosphate buffer, pH 7.0. One hundred μL diluted sample was mixed with 2.9 ml substrate solution (0.1 ml 30% H_2O_2 in 50 ml 0.05 M phosphate buffer, pH 7.0) in a quartz cuvette at room temperature. Absorbance was followed at 240 nm and units of activity were defined as 3.45/time required for absorbance to decrease from 0.45 to 0.40.

Protein determinations were made with a commercially available kit (Pierce BCA protein assay reagent, Rockford, Illinois) based on the biuret reaction with bicinchoninic acid.[39] Two gels from the group exposed only to subarachoid agarose and OxyHb were analyzed by high pressure liquid chromatography for content of bilirubin. Following homogenization in 20 mM phosphate buffer (pH 7.5), methyl esters of bilirubin were extracted from supernatant fluid and bilirubin measured as described in Chapter Two (page 40). Hemoglobin content of these two gels was also measured by spectrophotometry.[4]

Pathologic Assessment

Following killing, the osmotic pump was removed and cut open to ensure that it was empty. Patency and intrathecal position of the catheter were checked to ensure the catheter was not blocked and that mechanical compression of cerebral arteries had not occurred. Brain and residual agarose gel were removed and photographed and the

circle of Willis dissected free. Residual agarose gel was weighed. Each brain and agarose gel was fixed in phosphate buffer containing 10% formalin for up to 4 weeks after which brains were sectioned coronally. Sections of cerebrum and residual clot from 2 monkeys in each group were stained with hematoxylin and eosin and examined by light microscopy.

Segments of right anterior cerebral artery from 2 animals randomly preselected from each group were placed in fixative (2.5% glutaraldehyde, 1% paraformaldehyde in Millonig's buffer, 0.12M, pH 7.4) at 4°C.[9] They were washed in Millonig's buffer, postfixed in 1% OsO_4 for 1 hour at room temperature, and dehydrated in a graded series of ethanol solutions. They were embedded in Araldite (Marivac Ltd., Halifax, Nova Scotia) and viewed at 75 kV in a Hitachi H-7000 transmission electron microscope (Hitachi Corporation, Tokyo, Japan).

Data Analysis

Diameters of the following cerebral arteries were measured at predetermined points 6 times with a calibrated optical micrometer and a mean value calculated: extradural internal carotid artery, intradural internal carotid artery, MCA, anterior cerebral artery, vertebral artery, and basilar artery. Data were coded, entered into a computer, and edited. All data are expressed as mean ± standard deviation. For angiographic vessel diameters and physiologic variables, comparisons were made within groups between day 0 and day 7 by paired t-test and between groups at each time period by analysis of variance followed by Scheffe's multiple comparison procedure. Concentration of MDA, weight of residual gel, and activities of catalase, protein, and SOD were compared using analysis of variance followed by Scheffe's multiple comparison procedure. Level of significance for all comparison tests was $p < 0.05$.

RESULTS

Comparisons within groups between day 0 and day 7 showed heart rate increased significantly ($p < 0.05$) in the OxyHb group and weight decreased significantly ($p < 0.05$) in animals receiving placebo (Table III-1). Comparisons between groups by analysis of variance at baseline revealed animals which had subarachnoid placement of OxyHb had significantly greater weight on day 0 ($p < 0.05$). At day 7 there were no significant differences in these variables. None of these changes were large or likely to affect angiographic vessel diameters.

Preliminary Experiments

Hemoglobin was released from the gel with a half life of 21 hours (Fig. III-2). Incubation of an osmotic pump *in vitro* confirmed release of SOD and catalase was constant over 7 days, corresponding to a pumping rate of 10 μL/hr (Table III-2). Study *in vivo* showed continuously elevated levels of SOD and catalase in cerebrospinal fluid for 7 days after pump implantation (Table III-2).

Clinical Status, Cerebral Vasospasm

All monkeys remained well and no focal neurologic deficits developed. There were no differences in angiographic vessel diameters between groups at baseline. At day 7, analysis of variance showed right MCA diameter was significantly different from control in all three groups exposed to OxyHb, although there were no pairwise differences ($p < 0.05$)(Table III-1). When comparisons were made within groups between baseline and day 7, monkeys which had subarachnoid agarose gel placed did not develop significant arterial narrowing (Figs. III-3 and III-4). In animals which underwent placement of OxyHb-agarose gel only, there was significant reduction in calibre of right MCA (41% ± 8%, $p < 0.005$)(Figs. III-3 and III-5). In animals treated with SOD and catalase, there was significant VSP of right extradural internal carotid artery (28% ± 8%, $p < 0.001$), right intradural internal carotid artery (29% ± 11%, $p < 0.001$), right MCA (27% ± 12%, $p < 0.005$), and right anterior cerebral artery (24% ±

4%, $p < 0.05$)(Figs. III-3 and III-6). Similar VSP developed after administration of placebo with significant reduction in right extradural internal carotid artery (24% ± 15%, $p < 0.05$), right intradural internal carotid artery (16% ± 12%, $p < 0.05$), and right MCA (26% ± 13%, $p < 0.05$)(Figs. III-3 and III-7). In animals receiving intrathecal SOD plus catalase or placebo, mild but significant decreases in diameter of left extradural internal carotid artery also developed (15% ± 9% and 11% ± 10%, $p < 0.05$, respectively).

Biochemical Measurements

Concentration of MDA in CSF aspirated from cisterna magna on day 7 in monkeys in each group is shown in Table III-3. The standard line correlating MDA concentration with peak height (Fig. II-21) and chromatograms which were similar to those obtained from samples in this experiment (Fig. II-22) are provided. No MDA was detected in CSF of animals exposed to subarachnoid agarose. Malondialdehyde was detected in equal amounts in each group which had subarachnoid OxyHb placed. Catalase activity was not detected in CSF aspirated from cisterna magna on day 7. Superoxide dismutase activity was present in low levels in CSF aspirated from cisterna magna on day 7 in all groups except, as one might expect, in animals which received intrathecal SOD plus catalase in which there was elevated activity (not significant). Protein concentration in CSF on day 7 did not differ significantly between the 4 groups. A standard line used for protein determinations is included (Fig. III-8). Bilirubin was not detected in one agarose-OxyHb gel on day 7. A second gel contained 7 ng of bilirubin, equivalent to a concentration of 2 μM in the gel. Chromatograms demonstrating the bilirubin elution profile and a standard line are provided in Chapter Two (Figs. II-16 to II-19). These two gels were found on day 7 to contain 4% and 10% of their original content of OxyHb.

Pathologic Examination

All pumps were empty on day 7 and there were no cases of catheter blockage, dislodgement, or impingement upon cerebral arteries. In animals which had subarachnoid agarose gel, clear agarose remained in the right sylvian fissure, right middle cranial fossa, and right anterior cranial fossa on day 7. In all other animals, green agarose gel was found in a similar location on day 7. There were no significant differences between groups in weight of residual gels at day 7 (Table III-3). Histologic examination of gels did not show evidence of hematomas or red blood cells (Fig. III-9). Agarose gels alone did not induce inflammation whereas gels containing OxyHb were surrounded by patchy subacute inflammatory exudate consisting of macrophages and polymorphonuclear leucocytes. The reaction was mild in all cases although inflammation was more diffuse and intense in animals which had subarachnoid placement of OxyHb and agarose only. Intrathecal injection of enzymes or placebo slightly reduced inflammation.

Light microscopy of brains and basal meninges from animals exposed to agarose were entirely normal. After placement of subarachnoid agarose containing OxyHb, a mild patchy infiltrate of polymorphonuclear and macrophage cells was observed in meninges of the right sylvian fissure. Erythrocytes were not present and differences were not discernable between animals exposed to OxyHb alone or in combination with enzyme treatment or placebo (Fig. III-9).

Transmission electron microscopy of anterior cerebral arteries of animals exposed to agarose were normal. Arteries exposed to gels containing OxyHb exhibited marked convolution of tunica intima and internal elastic lamina and endothelial cell vacuolation and desquamation (Fig. III-10). There was occasional loss of interendothelial cell tight junctions. In tunica media, extensive smooth muscle contraction and vacuolation were present. Small vacuoles surrounded by single membranes and containing cellular debri were present in addition to vacuoles surrounded by double membranes. Smooth muscle necrosis was observed although there was no intimal thickening, medial fibrosis, fragmentation or duplication of internal

elastic lamina, or infiltration of tunica media by inflammatory cells. Occasional polymorphonuclear leucocytes and macrophages were present in superficial areas of tunica adventitia. Similar changes were observed in arteries from animals given intrathecal SOD plus catalase or placebo. No differences were noted in frequency or severity of changes between the 3 groups exposed to OxyHb.

DISCUSSION

Oxyhemoglobin was combined with agarose to produce a gel which slowly released OxyHb into CSF, simulating hemolysis of subarachnoid erythrocytes after SAH. This caused VSP with angiographic distribution, severity, and morphologic features within cerebral arteries which are identical to those occurring after placement of clotted whole blood in the monkey subarachnoid space.[9,10] There seems little doubt that OxyHb is the cause of cerebral VSP.[15,48] Intrathecal administration of SOD and catalase failed to alleviate VSP due to OxyHb. These results, therefore, do not support a role for free radicals in genesis of arterial narrowing due to OxyHb. Alternatively, only one dosing regimen was tried (albiet continuously administered), and it is possible the dose of one or both agents was inappropriate. Another proposed mechanism of action of OxyHb is its metabolism to bilirubin,[7] another potential spasmogen. Gels, however, contained bilirubin in amounts far too low to induce VSP. Previous work also concluded that bilirubin was not important.[25]

These findings are inconsistent with evidence *in vivo* that OxyHb causes VSP through generation of injurious oxygen free radicals. Subarachnoid hemorrhage is associated with presence of lipid peroxides in CSF [41] and patients or experimental animals with VSP have higher levels of lipid peroxides than do subjects with SAH but no VSP.[1,19,21,33,34,36,47] It has been demonstrated that the already minimal CSF levels of SOD, catalase, and glutathione peroxidase are depleted after SAH, permitting lipid peroxidation to proceed unchecked.[33,34,47] In addition, large doses of lipid peroxides of arachidonic acid cause prolonged arterial narrowing after injection into cisterna magna of dogs.[37] An inhibitor of iron-dependent lipid peroxidation, U74006F, significantly diminished VSP after SAH in monkeys [18,41] and rabbits.[46,51] Oxyhemoglobin injections into dogs [44] and monkeys (see Chapter Two) were also associated with lipid peroxidation.

Trials of free radical scavengers and OxyHb *in vivo* have not been performed. There are, however, several reports of beneficial effects of 1,2-bis(nicotinamide)-propane on VSP after single and double blood injection into cisterna magna of dogs.[2,30] This scavenger also underwent a double-blind, placebo-controlled clinical trial in humans with SAH.[35] Treatment significantly reduced angiographic VSP and delayed neurologic deficits. Nakagomi et al found MCI-186, a free radical scavenger, significantly attenuated VSP in a two-hemorrhage dog model.[29] Ohue investigated effects of SOD plus catalase on VSP in a double-hemorrhage dog model.[30] Treatment "slightly reduced" basilar artery narrowing but had no effect on CSF MDA levels.

Experiments *in vitro* using free radical scavengers demonstrated that 1,2-bis(nicotinamide)-propane, α-tocopherol, and 1,4-diazabicyclo[2,2,2]octane prevent contractions induced by OxyHb in exposed cat basilar arteries.[11,17] Investigations with SOD and catalase on isolated vascular preparations have produced conflicting results. Kamiyama and coworkers found SOD and catalase were effective inhibitors of OxyHb-induced contraction of cat basilar artery exposed *in situ*.[17] Fujita et al blocked OxyHb induced contraction of dog basilar arteries with catalase but not with SOD.[11] Wellum et al and others found SOD and catalase did not prevent OxyHb from constricting dog basilar artery *in vitro*.[31,49] Electrophysiologic studies of isolated rat cerebral artery smooth muscle cells exposed to OxyHb demonstrated activation of calcium-dependent potassium currents and cell death. These changes were prevented by catalase but not by SOD.[40]

In view of the evidence cited above in favor of free radical mechanisms in OxyHb-induced VSP, one wonders why this treatment failed. First, the dose used may

have been incorrect. Pilot studies showed high levels of SOD and catalase in CSF sampled from a catheter adjacent to the site of intrathecal administration. These doses were selected as this was the maximal amount of SOD which could be administered by one pump. An equal amount of catalase was included, although there is little information on effects of combinations of the two agents. Activity of SOD remained above 3.2×10^4 U/ml and catalase above 1.1×10^4 U/ml for 6 days. These activities are far higher than those observed in CSF after SAH in association with VSP, when activities are apparently insufficient.[33,34,47] Cerebrospinal fluid aspirated from cisterna magna on day 7 contained no catalase and only low levels of SOD. A large gradient in concentration is therefore present and it is impossible to know precisely the enzyme concentrations next to MCA. Malondialdehyde levels were not diminished with treatment. This could be interpreted as evidence that not enough free radical scavenging activity was present. It is also consistent, however, with the similar degree of VSP and attendant arterial wall damage in all 3 groups exposed to OxyHb. There is evidence that protection conferred by SOD is dose-dependent. Omar et al assessed effects of different doses of SOD on infarct size in rabbit and rat hearts.[32] Low doses of SOD produced dose-dependent reductions in infarct size whereas high levels of SOD exaccerbated ischemic damage. Effective doses depended on the species and experimental preparation. The authors found previous reports of failure of SOD treatment to decrease infarction could be explained on the basis of dose-response curves which they generated. No such data exist for VSP and it is impossible to know if other doses or combinations of SOD and catalase might be efficacious. Dose-response curves for the effect of SOD have rarely been generated. Only Steele et al reported no difference in efficacy of a variety of SOD doses in maintaining viability of rat cerebrovascular smooth muscle cells after exposure to OxyHb.[40]

Second, after SAH blood components, and, presumably hemoglobin, penetrate arterial walls through nutrient channels within tunica adventitia. Oxyhemoglobin alone can produce VSP equal in severity to that produced by whole blood and, therefore it is likely that erythrocyte membrane breakdown with production of lipid peroxides probably plays only a secondary role in VSP. Since VSP correlates best with the volume of subarachnoid blood [48] and erythrocyte membranes spontaneously undergo lipid peroxidation, levels of lipid peroxides will correlate with VSP. If free radical reactions are important, therefore, OxyHb may act directly on vascular smooth muscle, producing lipid peroxides in arterial wall. Superoxide dismutase has a lower molecular weight (32,000 daltons) than hemoglobin (64,000 daltons) and could enter the arterial wall to protect against such reactions if hemoglobin did not first completely clog nutrient channels. Catalase is a large tetrameric protein of molecular weight 232,000 and might not enter as easily. "Site-specific" free radical production could therefore go unchecked due to inability of these large enzymes to get to the site of radical production.[13]

Third, OxyHb may cause VSP by multiple mechanisms. Inhibition of endothelium-dependent relaxation,[28] augmentation of endothelin synthesis,[26] release of vasoactive eicosanoids,[31,43] and damage to perivascular nerves,[22,23] could constrict cerebral arteries by mechanisms independent of free-radical reactions.

Fourth, a small therapeutic effect might have been missed due to small numbers of animals employed. Placebo insignificantly decreased VSP to similar degree as treatment with SOD plus catalase. This could be due to nonspecific free radical scavenging effect of protein, although protein concentration did not correlate with protection from ischemia in myocardium.[32] It is more likely the effect was due to increased washout of OxyHb from the gel due to infusion of fluid from the pump. It is well known that removal of blood clot laden with OxyHb, either by washing,[50] or with tissue-type plasminogen activator,[10] will diminish VSP.

These results do add to a body of literature documenting failure of SOD and catalase to alleviate processes purported to be due to free radicals. There is, for example, conflicting literature on efficacy of anti-free radical therapy for cerebral ischemia [16,38] and following ischemia and reperfusion in the heart.[6,32] As discussed

above, methodologic problems, including use of single doses, necessitates further work before arriving at definitive conclusions.

In summary, OxyHb-agarose gel was used to produce VSP in monkeys. Superoxide dismutase plus catalase, at doses employed in this study (which give CSF levels shown in Table III-2) did not prevent VSP due to OxyHb. Therefore, free radical mechanisms may not be critical to development of or may not be the sole pathogenetic process in cerebral VSP. Alternatively, this particular dose regimen of SOD and catalase may not effectively scavenge the particular free radicals which cause VSP.

Table III-1: Baseline (Day 0) and Day 7 Values for Physiologic Variables and Angiographic Vessel Diameters, by Group.[a]

	Agarose		Agarose + OxyHb		Agarose + OxyHb + Treatment		Agarose + OxyHb + Placebo	
Variable	day 0	day 7	day 0	day 7	day 0	day 7	day 0	day 7
n	2	2	3	3	6	6	6	6
body weight (kg)	2.2 ± 0.3	2.3 ± 0.1	2.6 ± 0.0	2.4 ± 0.1	2.3 ± 0.1	2.2 ± 0.2	2.5 ± 0.1	2.3 ± 0.1
MABP (mm Hg)	104 ± 30	79 ± 30	105 ± 8	86 ± 14	103 ± 31	82 ± 16	105 ± 16	99 ± 23
heart rate (min^{-1})	180 ± 17	156 ± 0	154 ± 8	185 ± 11	183 ± 6	183 ±17	185 ± 20	185 ± 18
PCO_2 (mm Hg)	45 ± 7	40 ± 1	39 ± 1	40 ± 0	40 ± 2	40 ± 2	40 ± 2	41 ± 4
PO_2 (mm Hg)	99 ± 1	104 ± 2	101 ± 8	102 ± 6	99 ± 10	101 ± 10	99 ± 6	103 ± 8
Vessel diameter (mm)								
C3, R	1.65 ± 0.36	1.67 ± 0.48	1.43 ± 0.26	1.43 ± 0.26	1.79 ± 0.20	1.28 ± 0.10	1.73 ± 0.17	1.31 ± 0.26
C3, L	1.75 ± 0.20	1.55 ± 0.39	1.55 ± 0.39	1.60 ± 0.26	1.81 ± 0.12	1.50 ± 0.09	1.77 ± 0.16	1.56 ± 0.16
C4, R	1.15 ± 0.23	1.15 ± 0.28	1.05 ± 0.09	0.71 ± 0.24	1.23 ± 0.12	0.86 ± 0.10	1.09 ± 0.11	0.91 ± 0.15
C4, L	1.14 ± 0.03	1.15 ± 0.23	1.03 ± 0.08	1.05 ± 0.11	1.12 ± 0.15	1.01 ± 0.10	1.08 ± 0.10	1.08 ± 0.14
ACA, R	0.94 ± 0	0.87 ± 0	0.70 ± 0.12	0.49 ± 0.25	0.85 ± 0.13	0.65 ± 0.08	0.81 ± 0.12	0.66 ± 0.17
ACA, L	1.10 ± 0.16	0.99 ± 0.51	0.78 ± 0.02	0.86 ± 0.11	0.82 ± 0.15	0.73 ± 0.08	0.89 ± 0.09	0.85 ± 0.13
MCA, R	1.05 ± 0.14	1.08 ± 0.13	0.94 ± 0.06	0.56 ± 0.04	0.90 ± 0.06	0.66 ± 0.12	0.94 ± 0.08	0.70 ± 0.16
MCA, L	0.97 ± 0.12	0.98 ± 0.35	0.90 ± 0.02	0.89 ± 0.06	0.96 ± 0.12	0.81 ± 0.08	0.90 ± 0.05	0.93 ± 0.08
VA, R	1.14 ± 0.24	1.09 ± 0.16	1.13 ± 0	0.97 ± 0.23	1.05 ± 0.17	0.79 ± 0.04	0.98 ± 0.24	0.92 ± 0.06
VA, L	1.16 ± 0.40	1.12 ± 0.28	0.78 ± 0.13	0.92 ± 0.06	0.91 ± 0.10	0.82 ± 0.02	0.98 ± 0.22	0.93 ± 0.15
BA	1.40 ± 0.29	1.33 ± 0.07	1.09 ± 0.06	1.11 ± 0.09	1.07 ± 0.18	0.96 ± 0.12	1.17 ± 0.20	0.97 ± 0.18

[a] Values are means ± standard deviations. OxyHb, oxyhemoglobin; MABP, mean arterial blood pressure; C3, extradural internal carotid artery; C4, intradural internal carotid artery; ACA, precommunicating segment of anterior cerebral artery; MCA, sphenoidal segment of middle cerebral artery; VA, vertebral artery; BA, basilar artery; R, right; L, left.

Table III-2: Catalase and Superoxide Dismutase Activities Released by Osmotic Pump *In Vitro* and *In Vivo*, by Day (n = 1 for each measurement, all activities are U/ml)

	Catalase Activity		Superoxide Dismutase Activity	
Day	*In Vitro*	*In Vivo*	*In Vitro*	*In Vivo*
0	-	0	-	1400
1	-	28200	-	78000
2	91000	24800	170000	25000
3	-	34500	-	18000
4	105000	31100	190000	65000
5	-	34500	-	69000
6	97000	11300	150000	32000

Table III-3: Weight of Residual Gel, Activity of Superoxide Dismutase, and Concentrations of Malondialdehyde and Protein in Cerebrospinal Fluid Aspirated from Cisterna Magna on Day 7, by Group.[a]

Group	n	Gel Weight (g)	Superoxide Dismutase (U/ml)	Malondialdehyde (nmol/ml)	Protein (mg/ml)
Agarose	2	0.53 ± 0.12	0.17 ± 0.12	0 ± 0	0.46 ± 0.22
Agarose + OxyHb	3	0.51 ± 0.11	0.26 ± 0.22	1.5 ± 0.9	0.35 ± 0.27
Agarose + OxyHb + Treatment	5	0.54 ± 0.16	7.7 ± 17	1.7 ± 1.9	0.28 ± 0.14
Agarose + OxyHb + Placebo	5	0.59 ± 0.19	0.37 ± 0.22	1.2 ± 1.0	0.20 ± 0.06

[a] Values are means ± standard deviations. OxyHb, oxyhemoglobin.

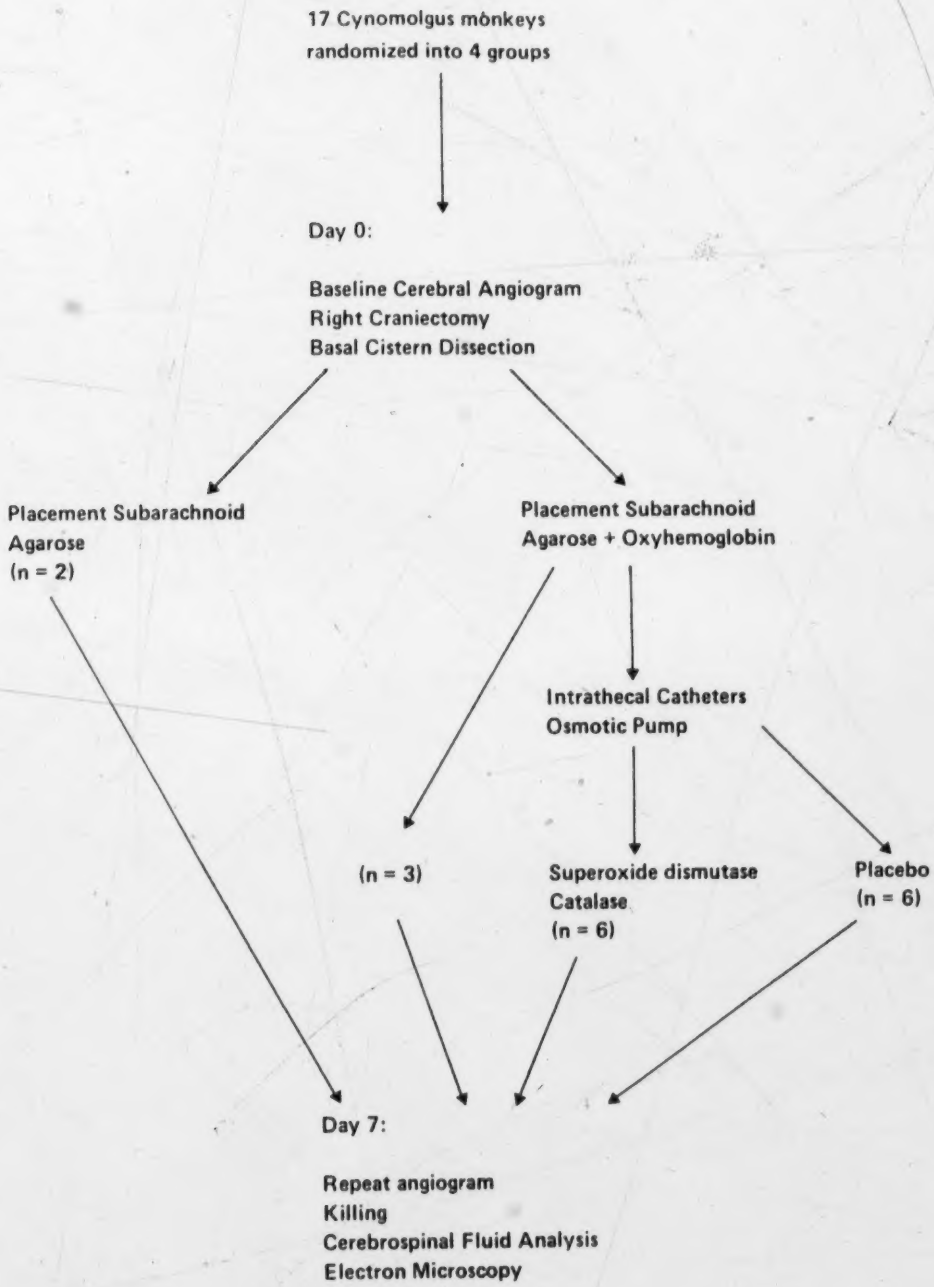

Figure III-1: Flow chart of experimental design employed in a randomized, controlled study of 17 monkeys with vasospasm induced by oxyhemoglobin and treated with free radical scavenging enzymes, superoxide dismutase and catalase.

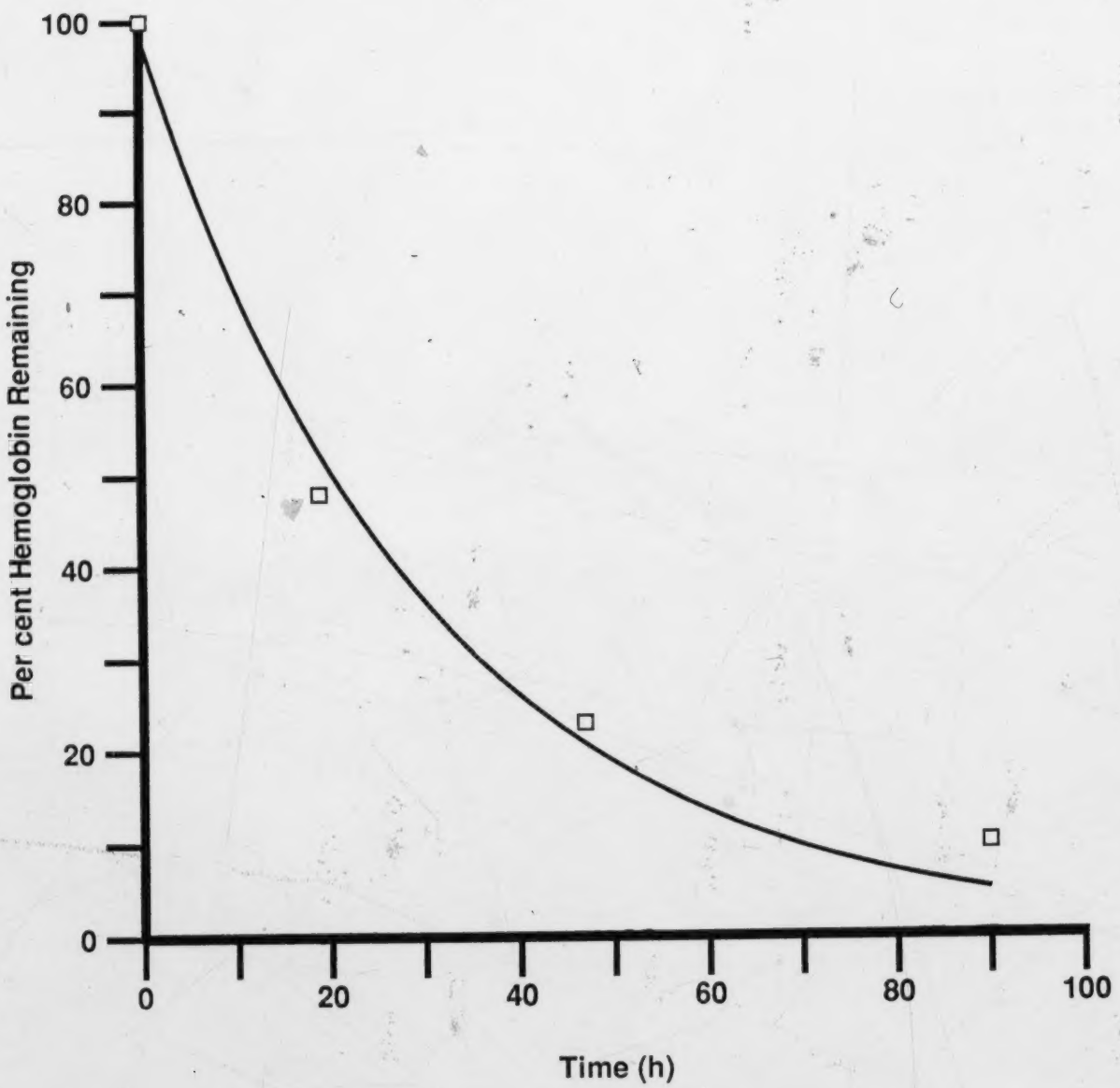

Figure III-2: A gel consisting of agarose and 350 mg hemoglobin was incubated in a buffer with composition similar to cerebrospinal fluid. Rate of release of hemoglobin from agarose into solution was calculated by measuring concentration of hemoglobin in solution and determining what per cent of total hemoglobin had been released from the gel. Graph plots per cent of hemoglobin remaining in gel (y axis) versus time (x axis), showing hemoglobin was released from gel with a half life of 21 hours.

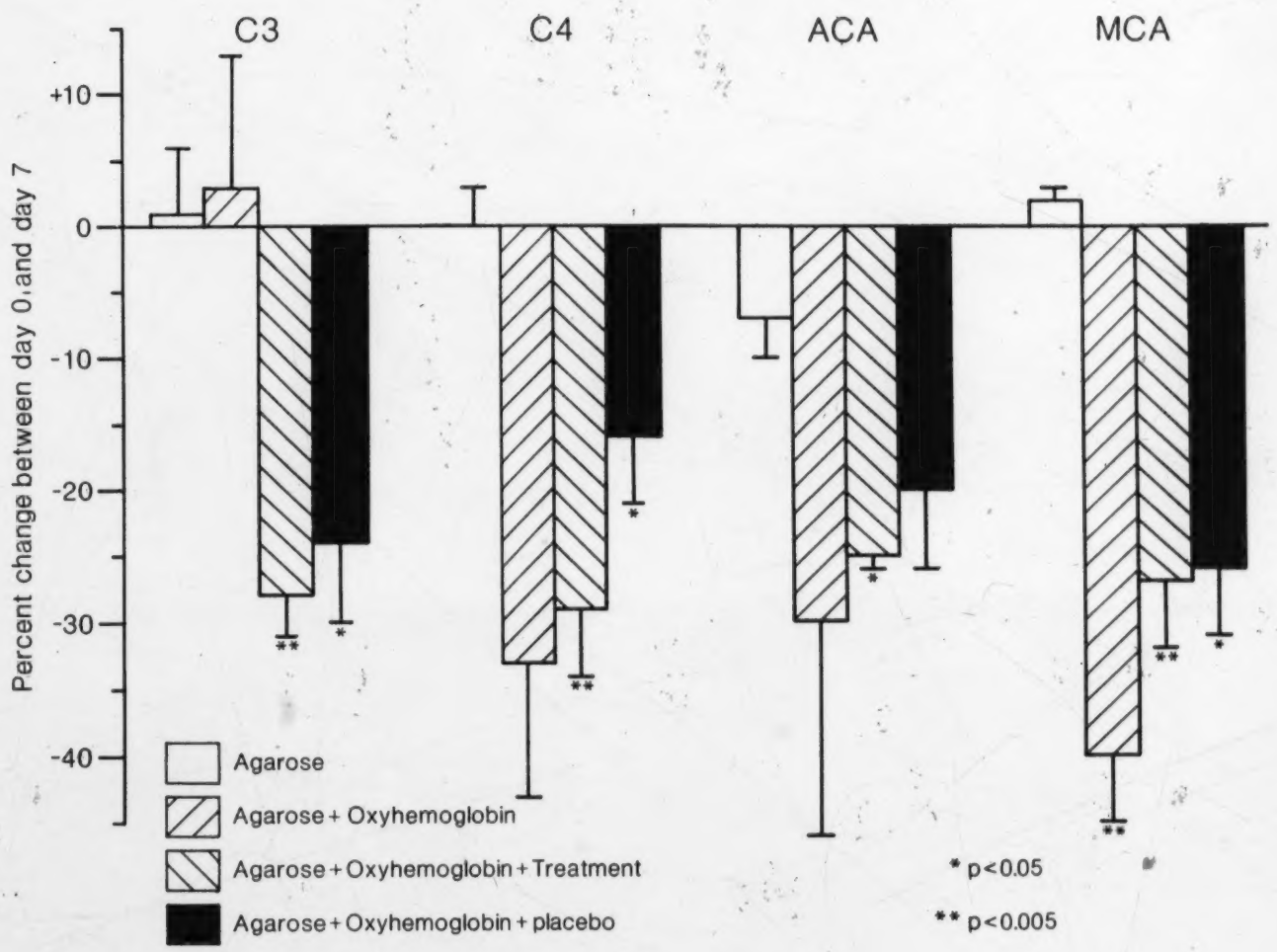

Figure III-3: Effect of subarachnoid placement of agarose (n = 2), agarose plus oxyhemoglobin (n = 3), agarose plus oxyhemoglobin plus intrathecal superoxide dismutase and catalase (treatment, n = 6), or agarose plus oxyhemoglobin plus placebo (n = 6) on right-sided cerebral arterial diameters, as assessed by comparison (by paired t-test) of angiograms taken on day 0 and 7 days after placement of subarachnoid compounds. Bars represent mean per cent reductions (± standard error). Significant reductions occurred in middle cerebral artery diameter after subarachnoid placement of oxyhemoglobin (41% ± 8%, $p < 0.005$). In animals treated with superoxide dismutase and catalase, there was significant vasospasm of extradural internal carotid artery (28% ± 8%, $p < 0.001$), intradural internal carotid artery (29% ± 11%, $p < 0.001$), middle cerebral artery (27% ± 12%, $p < 0.005$), and anterior cerebral artery (24% ± 4%, $p < 0.05$). Similar vasospasm developed after administration of placebo with significant reduction in extradural internal carotid artery (24% ± 15%, $p < 0.05$), intradural internal carotid artery (16% ± 12%, $p < 0.05$), and middle cerebral artery (26% ± 13%, $p < 0.05$).

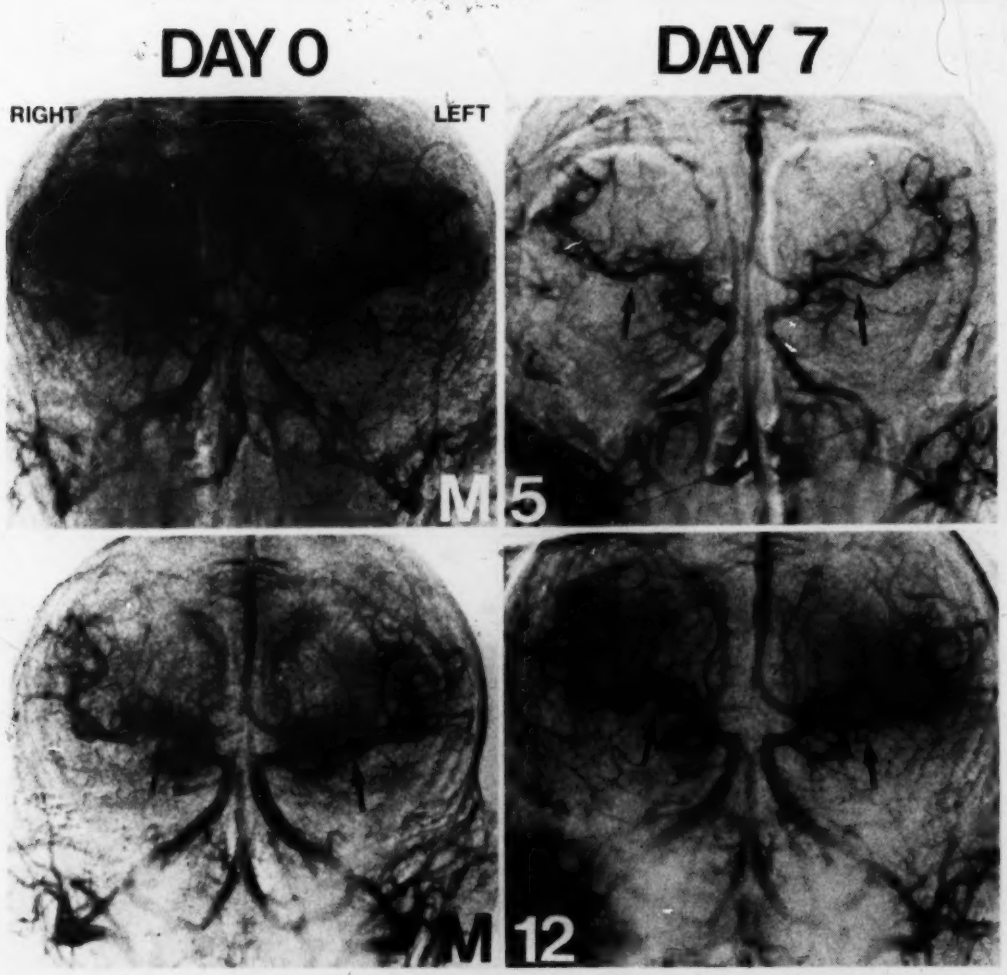

Figure III-4: Cerebral angiograms taken on day 0 and on day 7 for animals having subarachnoid agarose placed. There is no significant vasospasm of right-sided cerebral arteries on day 7. Arrows indicate middle cerebral arteries.

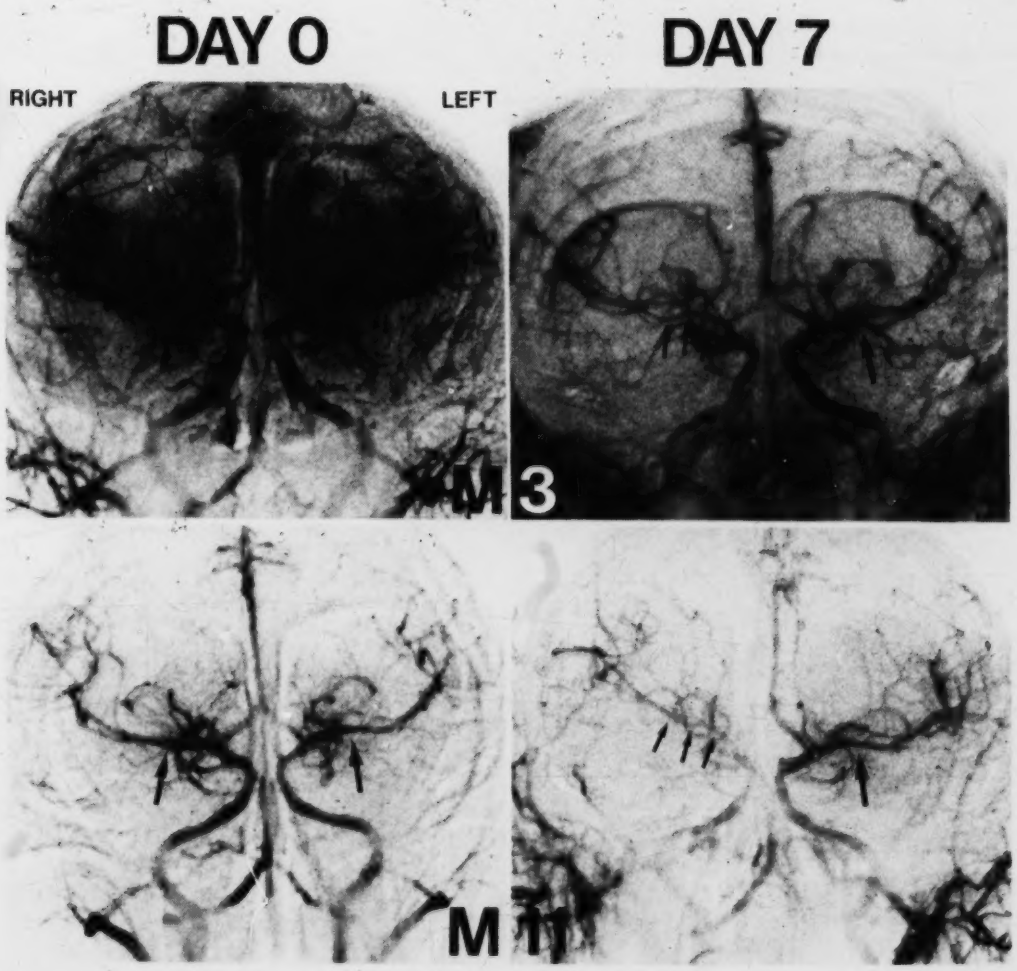

Figure III-5: Cerebral angiograms taken on day 0 and on day 7 for animals which had subarachnoid oxyhemoglobin-agarose gel placed. Note the presence of vasospasm of right-sided middle cerebral arteries on day 7. Arrows indicate middle cerebral arteries.

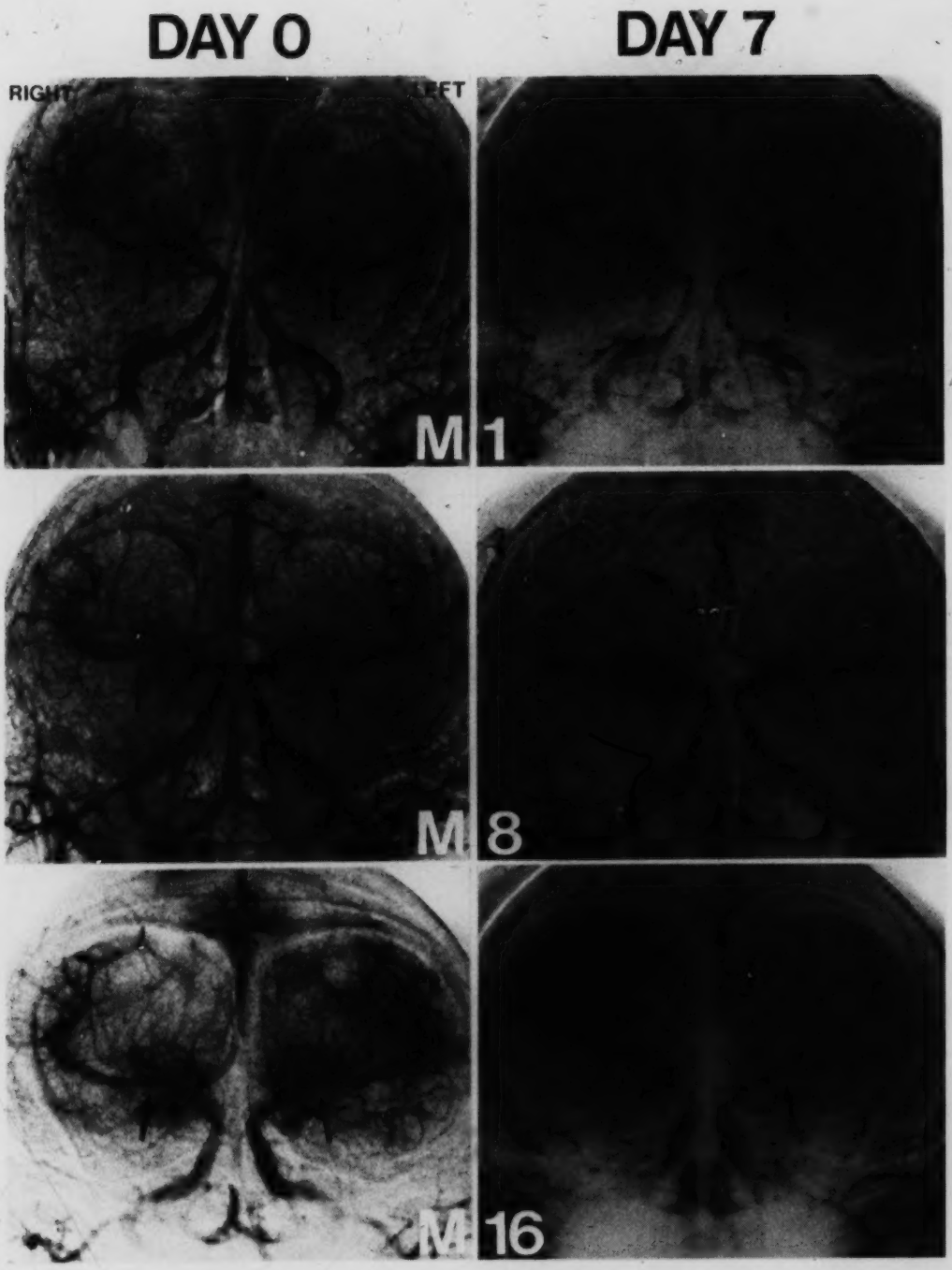

Figure III-6: Cerebral angiograms taken on day 0 and on day 7 for animals which had subarachnoid oxyhemoglobin-agarose gel placed and were treated with intrathecal superoxide dismutase and catalase. Despite treatment, there is vasospasm of right-sided cerebral arteries on day 7. Arrows indicate middle cerebral arteries.

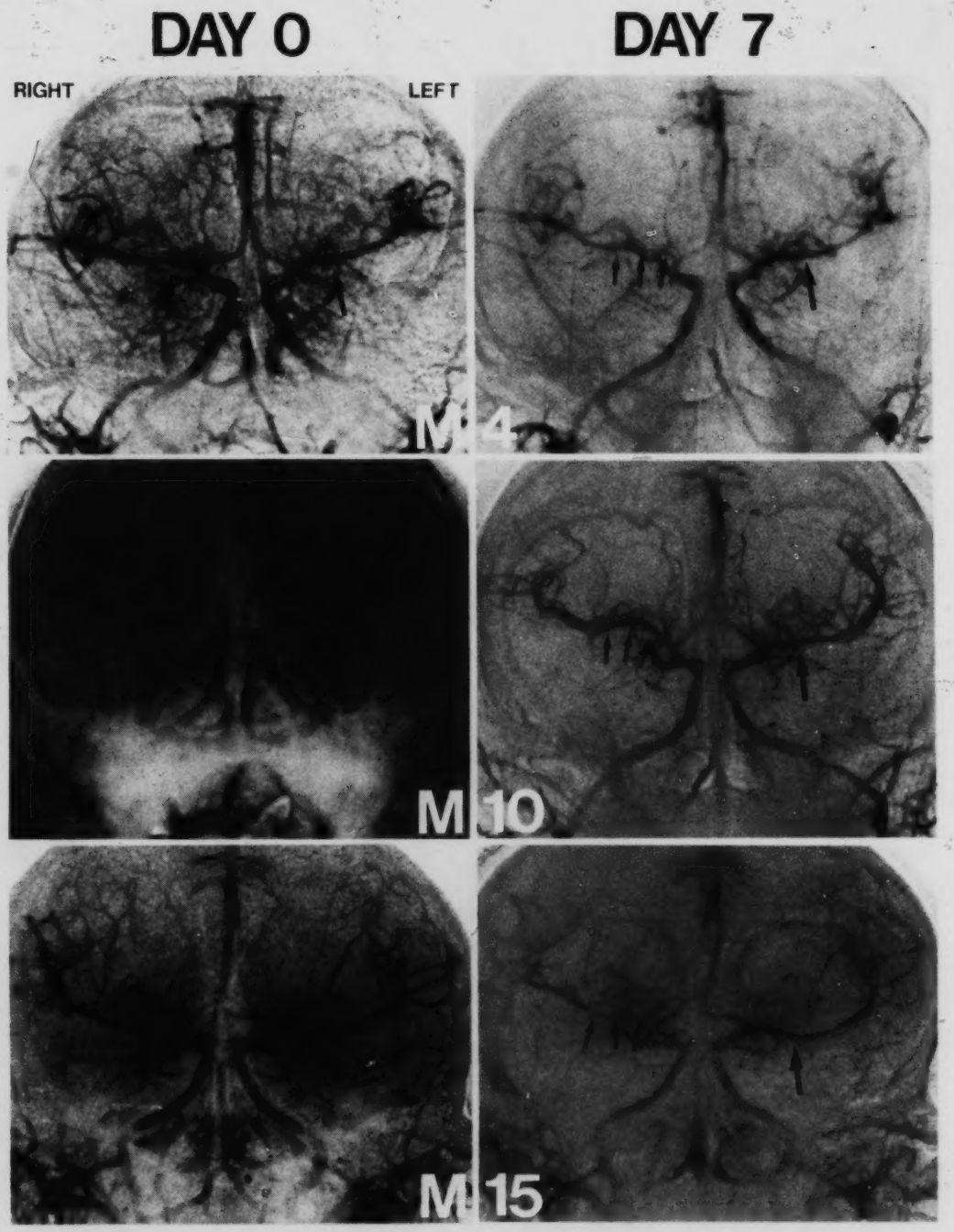

Figure III-7: Cerebral angiograms taken on day 0 and on day 7 for animals which had subarachnoid oxyhemoglobin-agarose gel placed followed by treatment with intrathecal placebo.. There is vasospasm of right-sided cerebral arteries on day 7. Arrows indicate middle cerebral arteries.

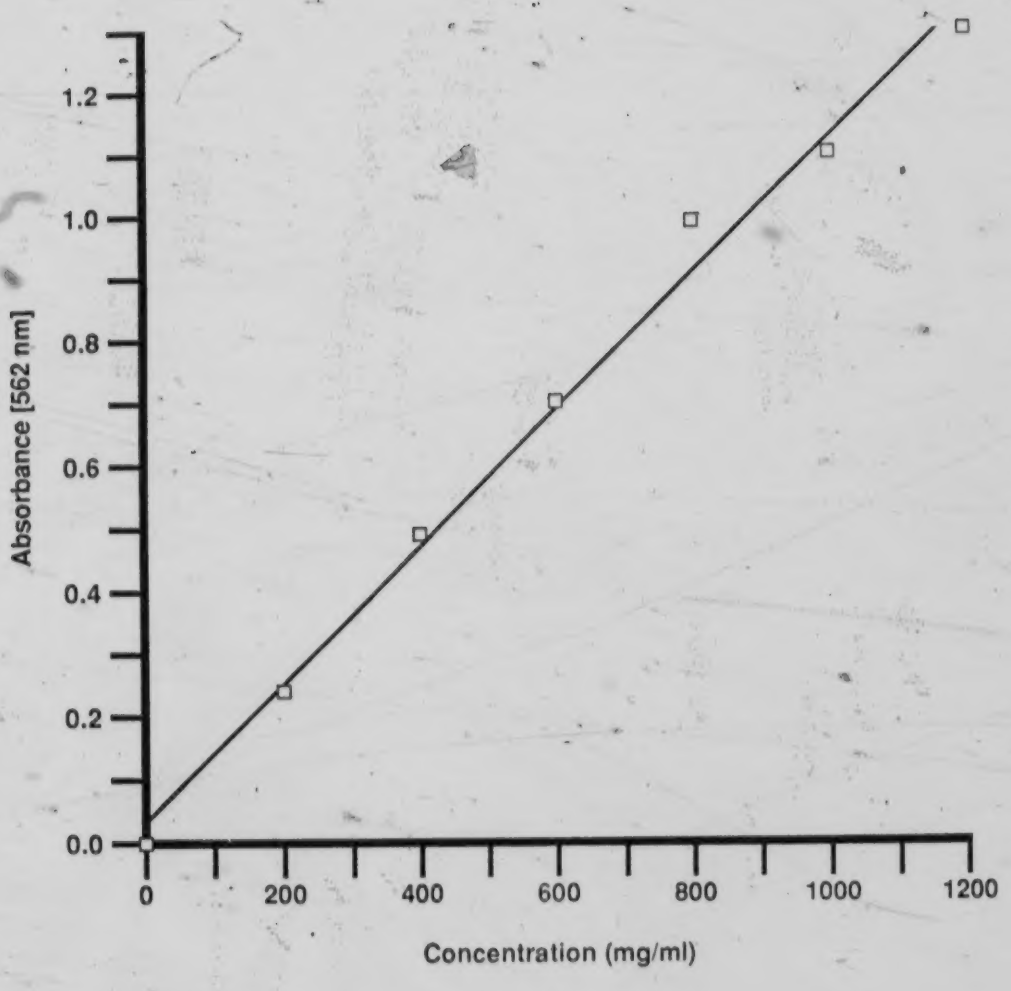

Figure III-8: Standard line for protein assay by buiret reaction with bicinchoninic acid. Absorbance at 562 nm (y axis) is plotted against concentration of bovine serum albumin (mg/ml) in solution ($r = 0.996$, $p < 0.0001$, by linear regression analysis).

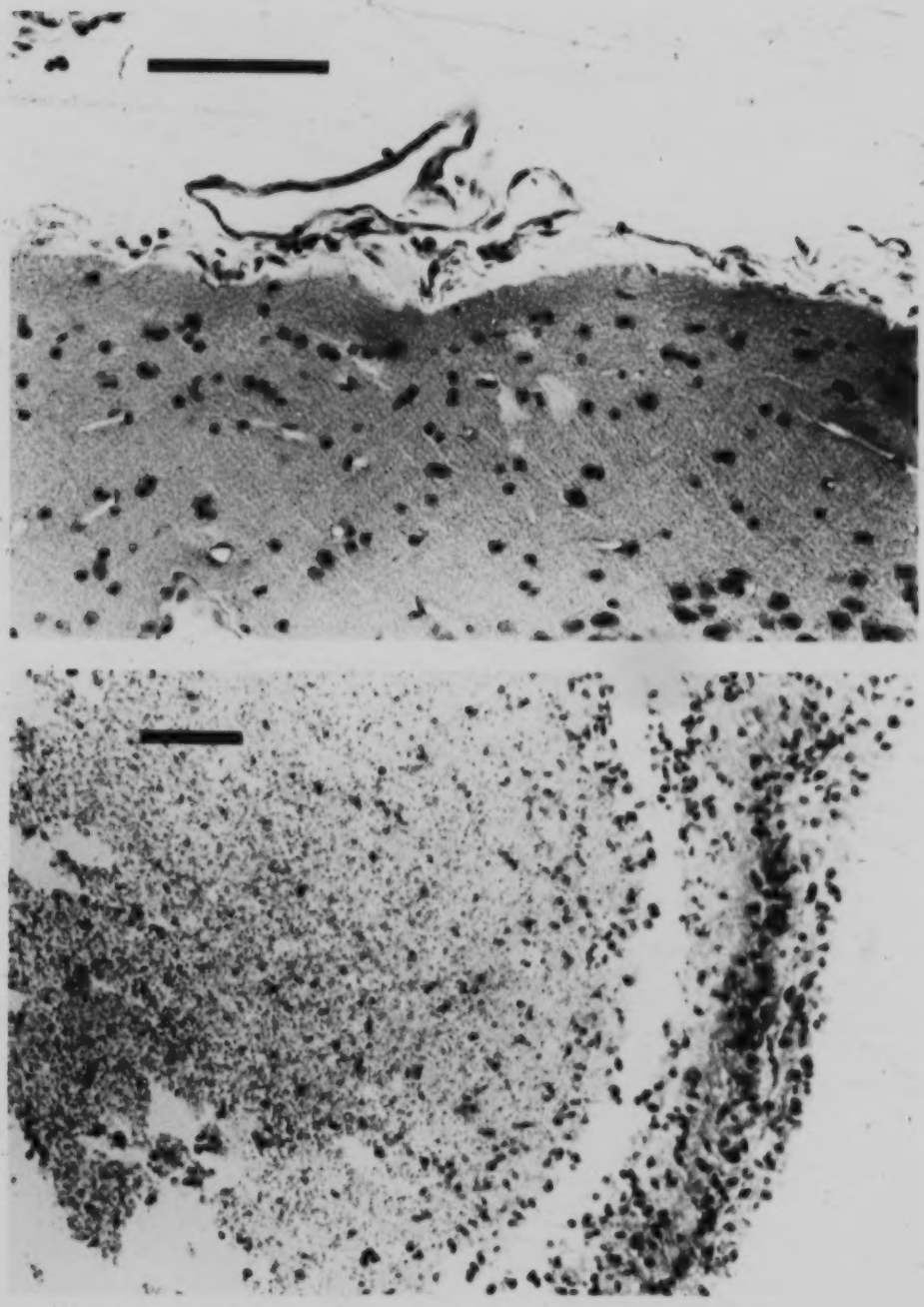

Figure III-9: Top: photomicrograph of right sylvian fissure of monkey exposed to subarachnoid agarose gel containing no oxyhemoglobin. The meninges and subarachnoid space are normal (hematoxylin and eosin, scale bar = 50 μm). Bottom: photomicrograph of mild inflammatory reaction over surface of oxyhemoglobin-agarose gel from an animal which did not receive intrathecal injections (hematoxylin and eosin, scale bar = 50 μm).

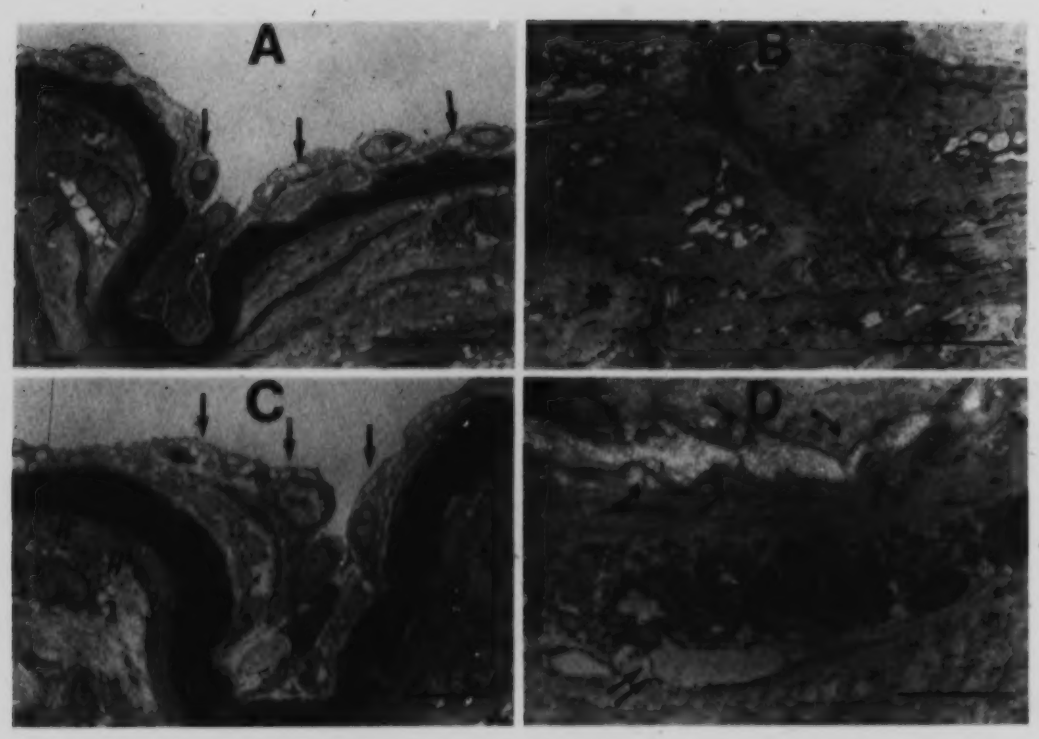

Figure III-10: Electron photomicrographs of anterior cerebral arteries from monkeys exposed to oxyhemoglobin-agarose gel. A: After exposure to subarachnoid oxyhemoglobin-agarose gel, there is vacuolation of endothelial cells (arrows), convolution of internal elastic lamina, and contraction and vacuolation of smooth muscle cells (double arrows)(scale bar = 10 μm). B: Medial smooth muscle shows contraction, and vacuolation after exposure to oxyhemoglobin-agarose gel. Most vacuoles represent swollen mitochondria (arowheads). One smooth muscle cell has decreased electron density (asterisk), indicative of necrosis (scale bar = 5 μm). C: Similar changes with endothelial cell vacuolation (arrows), folding of internal elastic lamina, and smooth muscle cell contraction and vacoulation (double arrows) are seen after placement of subarachnoid oxyhemoglobin-agarose and treatment with superoxide dismutase plus catalase (scale bar = 5 μm). D: Swollen mitochondria and vacuolation within contracted smooth muscle cell (double arrows) after exposure to subarachnoid oxyhemoglobin-agarose and treatment with placebo. Folding of the cell membrane, a feature of smooth muscle contraction, is visible (curved arrows)(scale bar = 2 μm).

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CHAPTER FOUR [a]
MORPHOMETRIC ANALYSIS OF MONKEY CEREBRAL ARTERIES EXPOSED *IN VIVO* TO SPASMOGENS

SUMMARY

Whether vasospasm (VSP) following subarachnoid hemorrhage (SAH) results from smooth muscle contraction or from arterial wall infiltration by cells and other material is subject to debate. Computer-assisted image-analysis was used to measure lumen area, total wall area, and area of tunica media plus tunica intima of cross-sections of monkey right middle cerebral arteries (MCA) exposed *in vivo*, for 6 days, to whole blood (n = 4), oxyhemoglobin (OxyHb, n = 5), methemoglobin (MetHb, n = 5), bilirubin (n = 5), mock cerebrospinal fluid (CSF, n = 6), and supernatant fluid from an incubated mixture of autologous blood and mock CSF (n = 5). Five control (left) MCAs from each group and 5 MCAs contracted *in vitro* with potassium chloride were measured. Significant VSP, as judged by comparison of angiograms taken at baseline and after 6 days of exposure to spasmogens, occurred in groups exposed to whole blood, supernatant fluid and OxyHb ($p < 0.05$). There was a significant correlation ($r = 0.58$, $p < 0.05$) between right MCA diameter on angiography and diameter calculated from lumen area. When compared to effects of mock CSF, OxyHb significantly increased total wall area. When right and left MCAs were compared within groups, total wall area increased similarly in every group with significant increases in groups exposed to mock CSF, OxyHb, and bilirubin ($p < 0.05$). No changes developed in area of tunica media plus intima, whether comparing right versus left MCAs within groups or right MCAs between groups. Contraction *in vitro* did not significantly increase total wall area or area of tunica media plus tunica intima. Light microscopy demonstrated inflammatory debri in the tunica adventitia of arteries from every group. This study shows that whole blood, OxyHb and supernatant fluid, which contains OxyHb, cause VSP. Increases in total wall area were not sufficient to account for luminal narrowing and therefore, changes such as cell proliferation and arterial wall fibrosis in the intima or media apparently do not contribute primarily to arterial narrowing of VSP. Vessel wall thickening which does occur is caused by increased tunica adventitia area only and is nonspecific in that it occurred after injection of substances not associated with VSP. The data are consistent with the hypothesis that OxyHb causes VSP (both angiographic and morphologic) by inducing muscle contraction in the media.

INTRODUCTION

Cerebral VSP following subarachnoid hemorrhage (SAH) may be due to exaggerated and prolonged smooth muscle contraction.[9,28] Vasospastic arteries, however, exhibit features not typical of physiologic vasoconstriction, including impaired relaxation responses to pharmacologic vasodilators,[4,15,24] stiffening of contractile and noncontractile components of the arterial wall,[15,30] and pathologic changes in smooth muscle and endothelial cells.[3,4,9,21,22,28] Alternatively, proliferation of cellular and connective tissue elements in the arterial wall after SAH could lead to arterial narrowing.[4,9] This hypothesis predicts that vasospastic arteries should have increased wall area compared to normal arteries.

We used computerized-assisted image processing to analyze arteries from a monkey model of VSP. Objectives of this study were to investigate whether VSP

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[a] A version of this chapter has been accepted for publication. Macdonald RL, Weir BKA, Grace MGA, Martin TP, Doi M, Cook DA: Morphometric analysis of monkey cerebral arteries exposed *in vivo* to whole blood, oxyhemoglobin, methemoglobin, and bilirubin. **Blood Vessels** 1991 (in press).

increases arterial wall area enough to account for luminal narrowing and to compare vessel diameters calculated from lumen areas measured by image-analysis with diameters obtained by angiography.

MATERIALS AND METHODS

Protocol

Forty female cynomolgus monkeys (Macaca fascicularis) weighing between 2.4 and 4.1 kg were assigned by restricted randomization to one of 5 groups of 8. Angiography, basal cistern dissection, placement of Ommaya reservoir and subarachnoid catheter for intrathecal injections, killing, and pathologic examination were carried out as previously reported (Chapter Two).[7,8,18] On day 0, each animal underwent baseline physical assessment and cerebral angiography. A right pterional craniectomy was performed and the basal cisterns were opened. An Ommaya reservoir was placed in the subcutaneous tissue and connected to a catheter coursing along the right MCA. On days 1 to 6, animals received intrathecal injections, twice a day, with one of the following solutions: 1. OxyHb, 2. MetHb, 3. bilirubin, 4. mock CSF, 5. supernatant fluid from an incubated mixture of autologous blood and mock CSF. On day 7, angiography was repeated and animals were killed. Five animals in each group were randomly selected for perfusion-fixation and morphometric analysis of both MCAs.

In addition to the experiments in which spasmogens were added via an Ommaya reservoir, 4 monkeys weighing between 2.9 and 4.7 kg were subjected to baseline cerebral angiography, basal cistern dissection, and placement of subarachnoid blood clot.[8] Angiography was repeated 7 days later and animals were killed and fixed by perfusion.

Injected solutions were of different color precluding blinding of the experimenter although interpretation of angiograms, computerized-image processing and data analysis were blinded. Protocols were evaluated by the Animal Ethics Review Committee of the University of Alberta. Care and surgery of animals were according to standards set by the Canadian Council on Animal Care.

Solution Preparation

Supernatant fluid was prepared on day 0 by drawing arterial blood from monkeys, mixing it with mock CSF (Elliott's solution B [6]) in a sterile container, and incubating the mixture at 37°C in the dark. Small volumes were removed twice each day, centrifuged, and 2 ml of supernatant fluid removed for injection. The remaining solution, along with 2 ml mock CSF, were returned to the sterile container. Oxyhemoglobin and MetHb were prepared from purified crystalline human hemoglobin (Sigma Chemical Company, St. Louis, Missouri) by the method of Martin et al.[19] Spectrophotometry showed the OxyHb solution to be 50% OxyHb and 50% MetHb and the MetHb solution to be over 99% MetHb. Pure bilirubin (Sigma Chemical Company, St. Louis, Missouri) was suspended in mock CSF immediately prior to injection. Sterility of injected solutions was confirmed periodically. Osmolarity and pH of bilirubin and hemoglobin solutions were 275 mOsm/L and 7.4, respectively. Osmolarity of supernatant fluid ranged from 273 to 293 mOsm/L and pH varied between 7.4 and 8.1.

Monkeys injected with supernatant fluid, OxyHb, and MetHb received a total of 600 mg hemoglobin, equal to that present in a 5 ml clot which was placed in animals in the whole blood group in this experiment and which is sufficient to cause VSP when placed in the cynomolgus monkey subarachnoid space.[8] The total amount of bilirubin injected (25mg) was that which would be produced by complete metabolism of 600 mg of hemoglobin.

Studies *In Vitro*

Four normal left MCAs were obtained from monkeys weighing between 2.1 and 2.5 kg which were killed but not perfused with fixative. Each MCA was procured within 10 minutes and placed in Krebs solution containing: Na^+; 132 mM, K^+; 5.9 mM, Ca^{2+}; 2.5 mM, Mg^{2+}; 1.2 mM, Cl^-; 122.7 mM, HCO_3^-; 25 mM, SO_4^{2-}; 1.2 mM, $H_2PO_4^-$; 1.2 mM, and glucose; 10 mM. Rings 3 mm long were cut from the artery and suspended on stainless steel wires in water-jacketed tissue baths in the same oxygenated solution at 37°C. Contraction was measured isometrically with Grass strain gauges (Model FTO3, Grass Instrument Co., Quincy, Massachusetts) and a polygraph (Model 7D, Grass Instrument Co., Quincy, Massachusetts). After equilibration for 1 hour, resting tension was set at 1 gram and contraction was induced with 60 mM potassium chloride in one ring from each artery, rings at resting tension serving as controls. After contraction, all baths were changed to contain fixative (3% glutaraldehyde and 1% paraformaldehyde in Millonigs buffer, 0.12 M, pH 7.4) in order to assess effects of fixative on arterial rings both at resting tension and during contraction.

Morphometric and Data Analysis

Five animals in each group given intrathecal injections and 4 animals exposed to whole blood underwent perfusion-fixation via a left ventricular cannula after opening the right atrium and cross clamping the descending aorta. Five hundred ml 0.9% NaCl under 110 mm Hg was followed by 0.5 L fixative (3% glutaraldehyde and 1% paraformaldehyde in Millonigs buffer, 0.12 M, pH 7.4 at 4°C). Both MCAs from these animals were prepared for morphometric analysis and light and electron microscopy (TEM, Hitachi H-7000 transmission electron microscope, Hitachi, Tokyo, Japan).

The sphenoidal segment of each MCA was removed from the brain and cut into 4 equal segments. Each segment was stored overnight in fixative solution at 4°C. Segments were then postfixed in 1% OsO_4 in Millonig's buffer at room temperature for 1 hour, dehydrated in a graded series of ethanol solutions, and embedded in Araldite (Marivac Ltd., Halifax, Nova Scotia). Sections from each arterial segment were cut orthogonal to the long axis of the artery and 5 sections from each segment were studied using a computerized-image processing system (Perceptive Systems, Inc. model PSICOM 323, League City, Texas).[20] The system consists of a television camera mounted on a Leitz microscope connected to the image processor and a LSI 11/73 microcomputer. The entire arterial cross-section was digitized as a 256 gray-level picture consisting of a 640 X 480 pixel array (Fig. IV-1). Edge thresholds were adjusted to define the arterial wall and area of lumen and area of vessel wall were computed. Edge thresholds were then adjusted to allow computation of area of tunica intima plus tunica media of each section. To ensure sections were perpendicular to the long axis of the artery, analysis was done on sections cut from several blocks at 5 degrees on either side of the perpendicular axis. All data were stored on magnetic tape.

Angiograms were interpreted by measuring each MCA 6 times with a calibrated optical micrometer. Mean values for each morphometric variable were obtained for each monkey by averaging the results for each of 4 segments examined from each artery. Means were calculated from these data for each variable and group. For studies *in vitro*, morphometric values were obtained by averaging values for all 4 arteries in each group. For correlation with angiographic MCA diameter, mean lumen area was computed for each MCA and diameter was then calculated using the formula: diameter = square root of [4 X area/π]. Linear regression analysis and calculation of Pearson's coefficient of correlation was performed separately on measurements for left and right MCA. Rank correlation and calculation of Spearman's correlation coefficient were also made for right and left MCAs.

For mean lumen area, total wall area, and area of tunica media and tunica intima, comparisons were made within groups between right and left MCA by paired t-test. In addition, since the control response is equivalent to the effects of mock CSF on right MCA, intergroup comparisons were made for each variable for both right and left MCAs by analysis of variance followed by, if significant variance was found,

Scheffe's multiple comparison procedure. Pairwise intergroup comparisons for each variable were made by an unpaired t-test by comparing data from each group to effects of mock CSF for both right and left MCA. For whole blood data, values were compared to values for right MCAs of mock CSF treated animals. All values are means ± standard deviations. Level of significance for all comparison tests was $p < 0.05$.

RESULTS

Vasospasm

Results of angiography are presented in detail elsewhere (Chapter Two)[18] and are only outlined here. Comparison of angiograms taken on day 0 and on day 7 showed significant reduction ($p < 0.05$) in diameter of right MCA in groups exposed to whole blood (50% ± 16%), supernatant fluid (26% ± 7%), and OxyHb (27% ± 5%)(Table IV-1). There were no significant changes in vessel caliber in groups receiving bilirubin, MetHb, and mock CSF, although a consistent but mild degree of arterial narrowing occurred in bilirubin-treated animals (13% ± 6%).

Morphometric Analysis

Linear regression analysis showed significant correlation ($r = 0.58$, $p < 0.05$) between angiographic diameter of right MCA and diameter of this artery calculated from lumen area (Fig. IV-2). For left MCA, there was no correlation (Fig. IV-3). Rank correlation gave similar results with Spearman's correlation coefficient for right MCA of 0.55 ($p < 0.001$) and for left MCA of 0.13 (not significant).

Analysis of variance of variables for right MCAs showed no significant differences between groups, although there was significant variance in right lumen area. Pairwise comparison of lumen areas of right MCAs showed significant reductions in lumen area in groups exposed to whole blood (73% ± 7%, $p < 0.005$), supernatant fluid (45% ± 10%, $p < 0.05$), and OxyHb (49% ± 9%, $p < 0.05$), results which correspond with those obtained angiographically (Table IV-2). Pairwise comparison of right MCA total wall area and area of tunica media plus intima showed only a significant increase in total wall area after injection of OxyHb (17% ± 36%, $p < 0.05$, Table IV-2).

Comparison of left MCA values for lumen area, total wall area, and area of tunica media plus tunica intima between groups showed no significant differences (Table IV-3).

Comparisons within groups between right and left MCA showed significant increases ($p < 0.05$) in total wall area occurred in groups injected with mock CSF (15% ± 6%), OxyHb (19% ± 22%), and bilirubin (23% ± 12%) although these were not of greater magnitude than those seen with MetHb (29% ± 40%) and supernatant fluid (22% ± 34%)(Table IV-1, Fig. IV-4). When adventitia was excluded and area of tunica intima and media measured, there were no significant changes detected (Table IV-1, Fig. IV-5).

All blocks cut at 5 degrees to either side of orthogonal sections had larger wall areas, confirming that sections were taken perpendicular to the long axis of the artery.

Studies *In Vitro:*

Contraction of arteries *in vitro* produced no significant increase in total wall area or area of tunica media plus tunica intima (Table IV-4). As one would expect for vessels examined under isometric tension, contraction produced no significant change in lumen area. At resting tension, fixation of arteries resulted in slight augmentation in contraction (increase of 0.25 g) in one artery. After contraction with potassium chloride, fixation resulted in no further changes in tension.

Microscopy:

Light microscopy of arterial sections showed macrophages, leucocytes, and inflammatory debris causing thickening of the tunica adventitia in right MCAs from animals which received intrathecal injections (Fig. IV-6). There was hemosiderin

staining in adventitia of animals injected with OxyHb, MetHb, and supernatant fluid. Bilirubin was deposited in adventitia of arteries from animals injected with bilirubin. No obvious subendothelial cell proliferation occurred in tunica intima. On TEM, changes associated with VSP were seen in arteries from animals exposed to whole blood, OxyHb, and supernatant fluid. These included vessel wall thickening, convolution of the internal elastic lamina, endothelial cell vacuolation, and vacuolation and necrosis of smooth muscle cells.

DISCUSSION

The results of this study suggest that VSP after placement of subarachnoid blood clot in monkeys does not significantly increase arterial wall area. Intrathecal injections of OxyHb and supernatant fluid also caused VSP as assessed by angiography and by morphometric analysis. Vasospasm due to OxyHb was associated with increased arterial wall area when compared to effects of mock CSF. When comparisons of wall areas were made within groups between right and left MCA, significant increases occurred after intrathecal injection of OxyHb as well as agents not associated with VSP (mock CSF). Since even mock CSF increased arterial wall area, surgical dissection of arachnoid from the MCA as well as effects of intrathecal catheters probably cause some increase in vessel wall area. This is consistent with results obtained from light microscopy of arteries which showed inflammatory cells and debri in the tunica adventitia of right MCAs from every group, although this reaction was generally more severe in animals exposed to OxyHb. Inflammation was confined to the tunica adventitia, an observation confirmed by image analysis which found area of tunica intima plus tunica media did not increase. Inflammation in response to surgical procedures and to intrathecal injection is not of major importance to VSP since whole blood, which caused the most severe VSP, caused only mild inflammation and did not significantly increase total wall area.

These findings preclude any role for proliferative vasculopathy in genesis of VSP. Using measurements from this study (for example, normal wall area of 0.15 mm^2, normal MCA internal diameter of 0.9 mm), a 20% increase in artery wall area would reduce internal diameter of the MCA to 0.87 mm assuming all area was added inside the vessel with its external diameter remaining fixed. To reduce lumen diameter by 50%, wall area would have to increase over 400%. Clearly, changes of this order of magnitude were not seen, whether comparing effects of spasmogens with contralateral MCAs or with effects of mock CSF. Vasospasm, therefore, must be due primarily to smooth muscle contraction and not proliferative vasculopathy. This is consistent with previous reports.[8,13] In humans dying after SAH, proliferative vasculopathy was not observed until one month after SAH, long after VSP usually resolves.[13,31] Intimal proliferation is a common response of arteries to injury and is preceeded by transient change in smooth muscle cell phenotype in tunica media.[5,27] Loss of immunoreactivity to α-actin in smooth muscle has been observed during VSP.[21,26] For intimal proliferation to contribute to lumen narrowing during VSP, this change in phenotype should preceed development of VSP. These changes are therefore consistent with the hypothesis that VSP is smooth muscle contraction which damages the arterial wall, resulting in a secondary response to injury, rather than from proliferative vasculopathy which then produces lumen narrowing.[9,28]

Although small increases in arterial wall area preclude proliferative processes as primary causes of VSP, they are consistent with theories that pathologic changes in vasospastic arteries, such as arterial wall fibrosis or inflammation, contribute to persistence of arterial narrowing.[3,4,15,24,30] Pharmacologic studies show vasospastic arteries are less compliant than normal [3,4,15,24,30] and this may be due to increases in arterial wall connective tissue in conjunction with decreases in smooth muscle cell volume, leading to severe arterial wall disruption but to only minor increases in wall area. Evidence for arterial wall fibrosis includes detection of type-V collagen, a form of collagen normally associated with wound healing, in vasospastic human arteries [28]

and increases in collagen and elastin in vasospastic arteries.[24,25] Failure of vasospastic arteries to reconstrict after angioplasty could imply that arteries constricted by VSP have severely damaged walls [12] (or that angioplasty damages them). Smooth muscle cells also become less compliant after SAH [15] suggesting that the contractile apparatus is temporarily fixed in a contracted state.

Data which do not support a role for these changes include the finding that whole blood, which produced the most severe VSP, did not increase total wall area. Oxyhemoglobin did cause an increase, but this was not of primary importance to VSP since narrowing of the lumen induced by OxyHb was less severe than that resulting from whole blood. Importance of finding type V collagen in vasospastic arterial walls is unclear since normal arteries also contain type V collagen.[23] Most microscopic and ultrastructural changes observed within vasospastic arteries can be produced by intense vasoconstriction.[1,9,14] In addition, before accepting that arterial wall fibrosis and inflammation (with concomitant loss of compliance) cause persistence of VSP, it should be shown that these changes, including collagen deposition, reverse as VSP resolves.

Our results are similar to those of Mayberg et al who used a piglet model of SAH to assess relative contributions of different components of the red blood cell (RBC) to VSP.[21,22] Vasospasm was assessed by measuring lumen area, wall thickness, and wall area of fixed cross-sections of vasospastic MCA and comparing them to contralateral MCA. Hemoglobin caused VSP which was associated with pathologic changes in the arterial wall, including convolution and fragmentation of internal elastic lamina, endothelial-cell distortion, pallor of smooth muscle cells, and degeneration of perivascular axons. Most RBC components increased wall thickness and wall area but only whole blood was associated with a significant increase in wall area (12.5% ± 15%). They concluded that all vasospastic activity of whole blood and of RBC cytosol was due to hemoglobin and that proliferative processes in the arterial wall did not contribute significantly to arterial narrowing during VSP.

Arterial diameters determined by image-analysis were found to correlate with diameters obtained by angiography and to give similar results to those of angiography, although significant variability was present between the two methods. Several authors correlated angiographic vessel diameters with diameters determined by morphometric methods, although few used computerized-image processing.[2,9,10,11,16,17,21,22] Lehman et al analyzed vasospastic arteries from rabbits and monkeys.[16] Excellent correlations were found between angiographic diameters and diameters obtained by tracing the internal elastic lamina on photographs of arterial cross-sections. The present study found substantial variation between the methods. At least one method must be biased in comparison to real vessel diameter since slope and y intercept of the correlation (Fig. IV-2) were not one and zero, respectively. Factors which potentially alter angiographic vessel diameters, including PCO_2, body temperature, and radiographic magnification, are carefully controlled for. Effects of fixation were examined *in vitro* and were found to be minimal. Source of the bias is thus unclear although despite the variability, both methods gave qualitatively similar results for vessel diameters.

Correlation between angiography and morphometry suggests that, if appropriate control arteries are measured, VSP in experimental models could be assessed solely based on morphometric measurements. Advantages of morphometric methods have been detailed [10,16,21] and include the ability to measure parameters such as wall area, wall thickness, and degree of vessel tortuosity. Disadvantages of morphometry are need for specialized apparatus and labor involved in analyzing numerous arterial cross-sections. The system used here was particularly useful because sections did not need to be photographed and manual measurements (for example, length of internal elastic lamina) were unnecessary.[20]

Vasospasm has often been shown to be associated with increased arterial wall thickness.[9,17,21,26] Wall thickness increases with simple vasoconstriction.[9,10,29] Findlay and colleagues measured wall thickness of vasospastic monkey MCAs and of MCAs contracted *in vitro* with prostaglandin $F_{2\alpha}$.[9] A 5-fold increase in thickness was found in both groups, confirming that large increases in wall thickness can be due simply to

vasoconstriction and do not necessarily indicate that wall hypertrophy or hyperplasia has occurred. Wall areas apparently increase very little during VSP.[21] It has usually been assumed that vasoconstriction does not increase wall area,[10] although this apparently has not been confirmed by measurements *in vitro*. Our data support this assumption. Since mass of arterial segments cannot change acutely *in vitro*, any increases, should they develop, would occur due to shortening of arterial rings as they contract, although a decrease in density of the tissue could also result in increased area. Vasospasm appears to be a form of vasoconstriction, and therefore, area of vessel wall containing smooth muscle (tunica media and intima areas) did not increase during VSP.

This study suggests that VSP due to whole blood or to OxyHb is primarily due to smooth muscle contraction. Pathologic changes associated with VSP are manifestations of damage incurred by severe and prolonged smooth muscle contraction. Further experimentation is needed to define what changes occur in arteries, what causes these changes, and how they contribute to known features of vasospastic arteries.

Table IV-1: Values for Angiographic and Morphometric Middle Cerebral Artery Diameter and for Lumen Area, Wall Area, and Area of Tunica Media and Tunica Intima, by Group and by side. All values are means ± standard deviations (R, right; L, left).

Group	Side	No. of Samples	Angiographic Diameter (mm)	Morphometric Diameter (mm)	Total Wall Area (mm^2)	Lumen Area (mm^2)	Area Media plus Intima (mm^2)
Supernatant	R	5	0.63±0.10	0.59±0.13	0.17±0.02	0.28±0.11	0.065±0.007
Fluid	L	5	0.80±0.08	0.78±0.17	0.13±0.05	0.49±0.18	0.070±0.022
Oxyhemoglobin	R	5	0.72±0.13	0.56±0.12	0.21±0.02	0.26±0.10	0.088±0.022
	L	5	0.82±0.11	0.81±0.08	0.17±0.03	0.51±0.09	0.082±0.017
Methemoglobin	R	5	0.80±0.10	0.75±0.14	0.26±0.08	0.44±0.15	0.090±0.034
	L	5	0.83±0.14	0.88±0.14	0.18±0.02	0.63±0.18	0.084±0.012
Bilirubin	R	5	0.73±0.17	0.64±0.14	0.20±0.04	0.32±0.13	0.077±0.005
	L	5	0.86±0.06	0.85±0.13	0.16±0.04	0.58±0.20	0.081±0.021
Mock Cerebrospinal	R	6	0.82±0.12	0.80±0.12	0.18±0.01	0.51±0.15	0.077±0.010
Fluid	L	6	0.88±0.06	0.85±0.12	0.15±0.02	0.58±0.12	0.076±0.008
Whole Blood	R	5	0.45±0.05	0.40±0.11	0.18±0.01	0.14±0.09	0.075±0.016

Table IV-2: Per cent change (means ± standard deviations) in measured variables between each group and mock CSF group for right middle cerebral arteries.

Group	Angiographic Diameter (mm)	Morphometric Diameter (mm)	Lumen Area (mm^2)	Total Wall Area (mm^2)	Area Media plus Intima (mm^2)
Supernatant Fluid	-23 ± 15 [a]	-26 ± 20 [a]	-45 ± 10 [a]	-5 ± 35	-16 ± 12
Oxyhemoglobin	-15 ± 16	-30 ± 20 [a]	-49 ± 9 [a]	+17 ± 36 [a]	+14 ± 20
Methemoglobin	-2 ± 14	-6 ± 18	-14 ± 21	+44 ± 32	+17 ± 28
Bilirubin	-11 ± 20	-20 ± 19	-36 ± 15	+11 ± 37	+1 ± 11
Whole Blood	-45 ± 22 [b]	-50 ± 24 [b]	-73 ± 7 [b]	0 ± 42	-3 ± 17
Mock Cerebrospinal Fluid	-	-	-	-	-

[a] $p<0.05$, unpaired t-test comparing value to value for mock cerebrospinal fluid group

[b] $p<0.005$, unpaired t-test comparing value to value for mock cerebrospinal fluid group

Table IV-3: Per cent change (means ± standard deviations) in measured variables between each group and mock CSF group for left middle cerebral arteries.

Group	Angiographic Diameter (mm)	Morphometric Diameter (mm)	Lumen Area (mm^2)	Total Wall Area (mm^2)	Area Media plus Intima (mm^2)
Supernatant Fluid	-9 ± 8	-10 ± 20	-17 ± 23	-13 ± 35	-8 ± 24
Oxyhemoglobin	-7 ± 15	-2 ± 12	-5 ± 15	+13 ± 24	+9 ± 17
Methemoglobin	-6 ± 11	+1 ± 14	+7 ± 12	+20 ± 27	+10 ± 13
Bilirubin	-2 ± 6	-2 ± 16	-2 ± 20	0 ± 32	+7 ± 17
Mock Cerebrospinal Fluid	-	-	-	-	-

Table IV-4: Values for lumen area, wall area, and area of tunica media plus tunica intima and per cent change in these variables (means ± standard deviations) for 4 left middle cerebral arteries *in vitro* under resting tension and after contraction with KCl. There are no significant differences in any measured variable.

Variable	Resting Tension	Contracted	Per Cent Change
wall area (mm^2)	0.15 ± 0.02	0.17 ± 0.02	+15 ± 20
lumen area (mm^2)	0.09 ± 0.01	0.11 ± 0.06	+23 ± 66
area of tunica media plus intima (mm^2)	0.082 ± 0.012	0.094 ± 0.022	+13 ± 13
morphometric diameter (mm)	0.33 ± 0.01	0.36 ± 0.10	+9 ± 28

Figure IV-1: Photographs showing computerized-image processing. Image of a MCA cross-section stained with toluidine blue (top) is digitized to a gray-level picture (center) to allow computation of wall area and lumen area. Edge thresholds are adjusted to exclude the tunica adventitia and define the tunica intima and tunica media (bottom) for calculation of area of these components of the arterial wall.

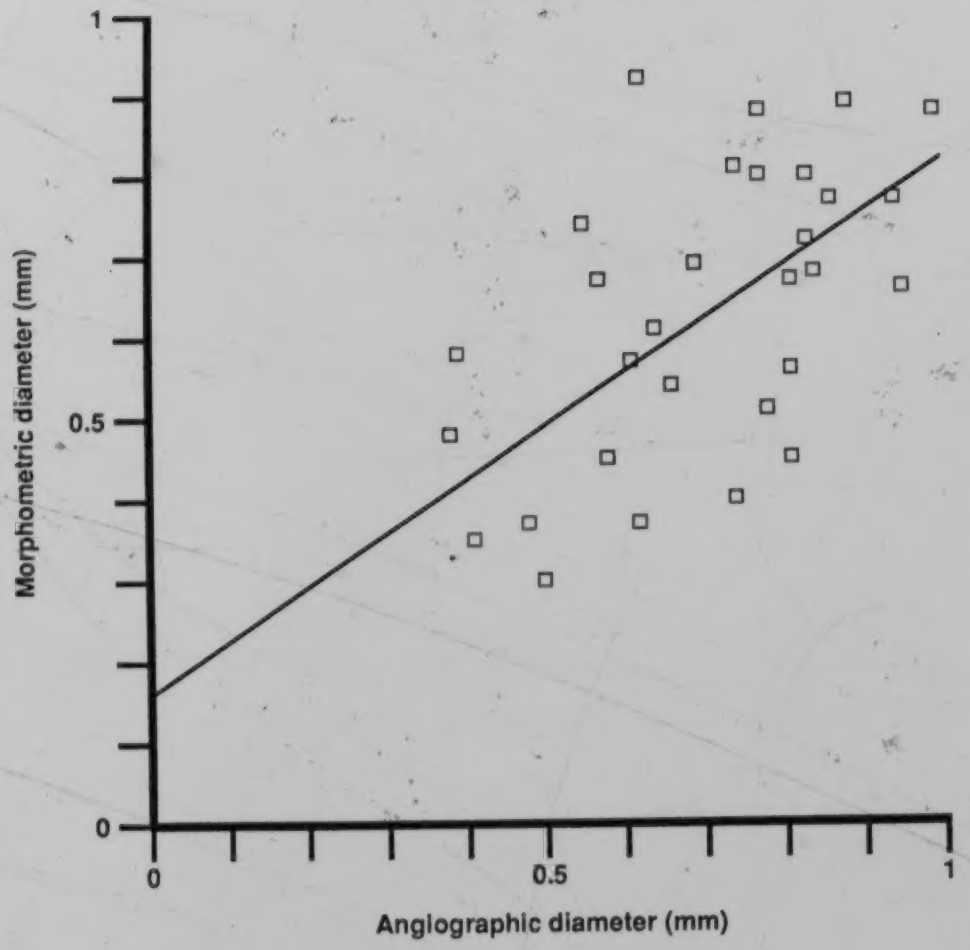

Figure IV-2: Graph showing linear regression analysis of right middle cerebral artery diameter as determined by image analysis (y axis) and right middle cerebral artery diameter from angiography (x axis). There is significant correlation ($r = 0.58$, $p < 0.05$) between these variables.

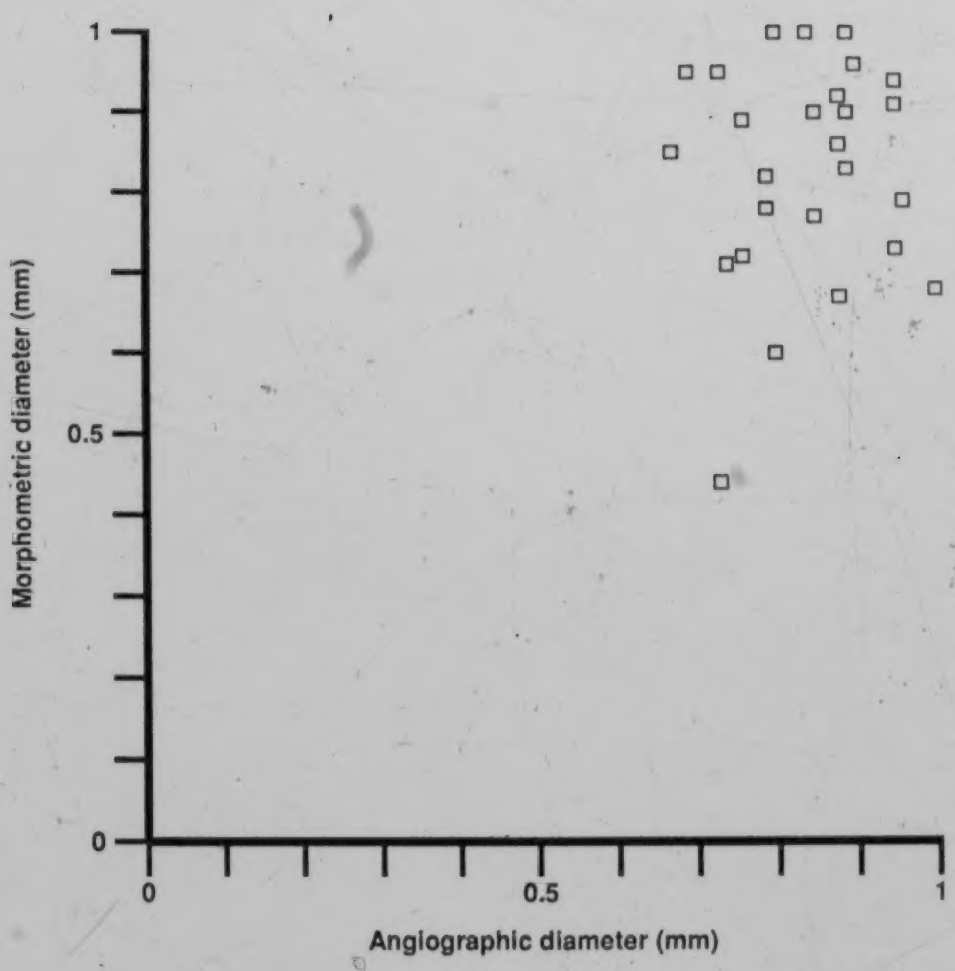

Figure IV-3: Graph showing linear regression analysis of left middle cerebral artery diameter as determined by image analysis (y axis) and left middle cerebral artery diameter from angiography (x axis). No significant correlation is present.

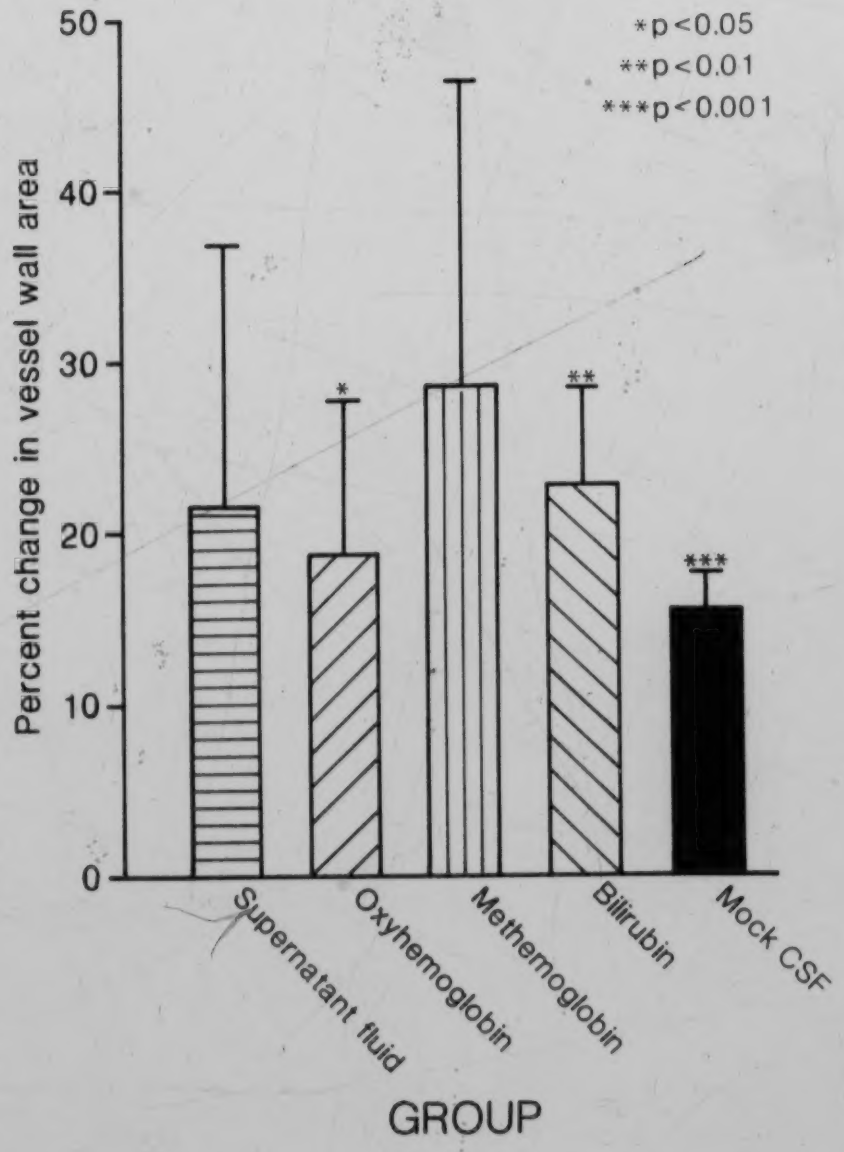

Figure IV-4: Effect of supernatant fluid from an incubated mixture of blood and mock cerebrospinal fluid (CSF)(n = 5), oxyhemoglobin (n = 5), methemoglobin (n = 5), bilirubin (n = 5), and mock CSF (n = 6) on middle cerebral artery wall area. Bars represent per cent change (± standard error) in total arterial wall area between right and left middle cerebral arteries within each group. Significant increases, as determined by paired t-test, developed after injection of oxyhemoglobin, bilirubin, and mock CSF.

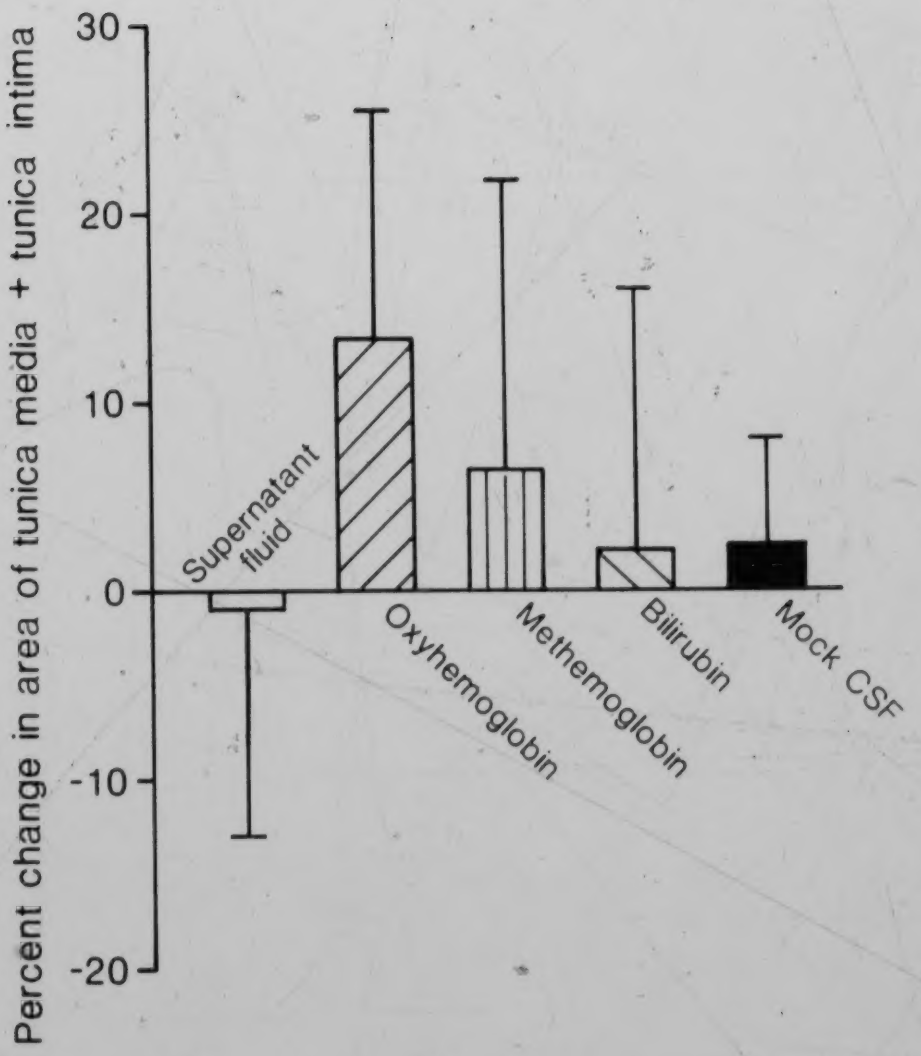

Figure IV-5: Effect of supernatant fluid from an incubated mixture of blood and mock cerebrospinal fluid (CSF)(n = 5), oxyhemoglobin (n = 5), methemoglobin (n = 5), bilirubin (n = 5), and mock CSF (n = 6) on area of tunica media plus tunica intima of middle cerebral arteries. Bars represent per cent change (± standard error) in area of tunica media plus intima between right and left middle cerebral arteries within each group. Analysis by paired t-test showed these changes were not significant.

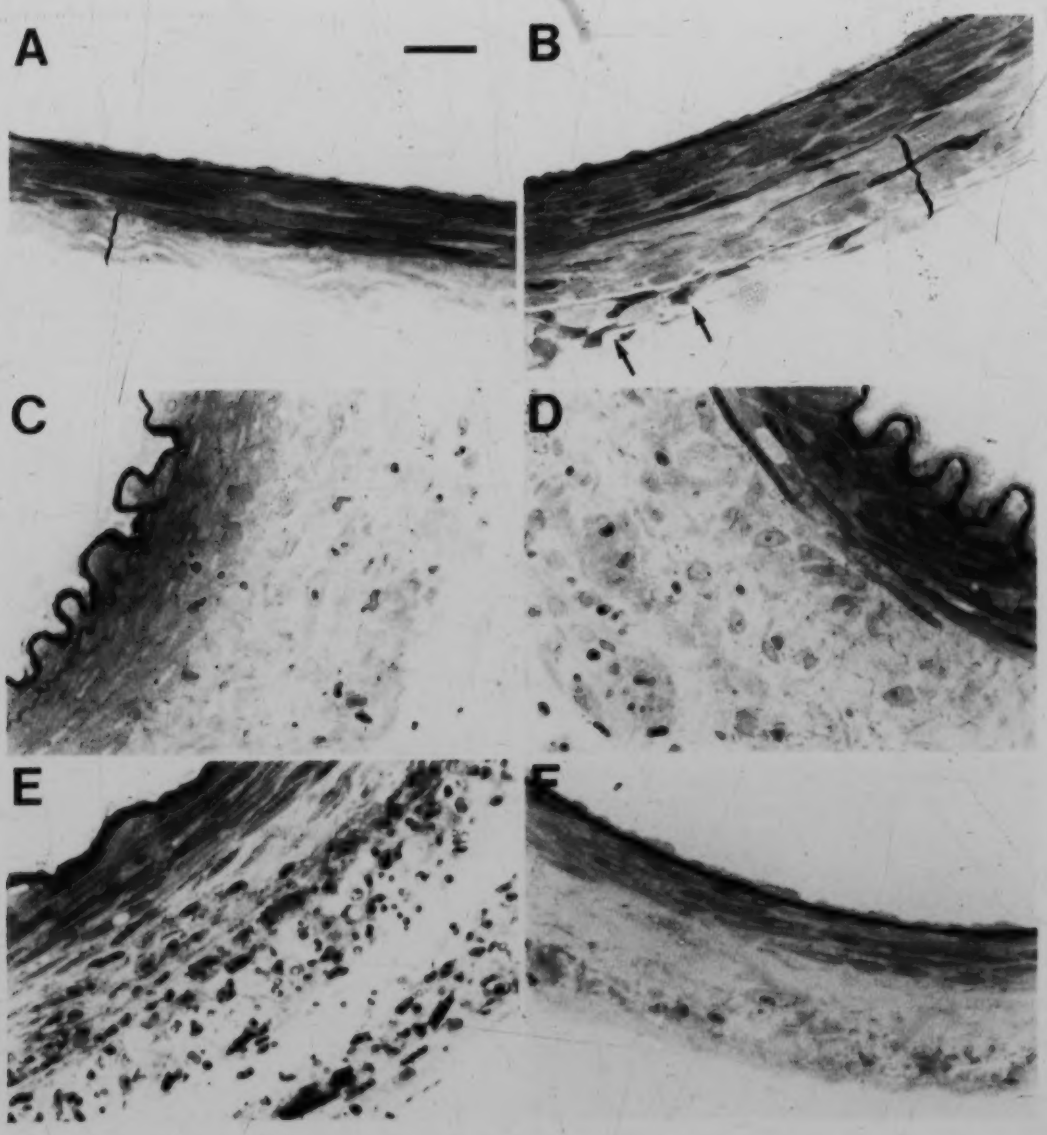

Figure IV-6: Photomicrographs of middle cerebral arteries from each group. A: normal left middle cerebral artery (MCA) from an animal injected with mock cerebrospinal fluid. The tunica adventitia (indicated by brace) is thin and acellular. B: After injection of mock cerebrospinal fluid, there is mild thickening of tunica adventitia with infiltration by inflammatory cells in the right MCA (arrows). Vasospasm of right MCAs after injection of supernatant fluid (C) or oxyhemoglobin (D) results in tremendous vessel wall thickening with infiltration of tunica adventitia with cells and inflammatory material. E: After injection of bilirubin, similar changes develop in right MCA with tunica adventitia thickening and cell infiltration, although there is less convolution of internal elastic lamina. After injection of methemoglobin (F), a section of right MCA shows similar inflammatory change in the tunica adventitia, although there is no folding of the internal elastic lamina (all sections stained with toluidine blue, Scale bar on A [= 100 μm] applies to all photomicrographs in illustration).

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CHAPTER FIVE [a]
SCANNING ELECTRON MICROSCOPY OF NORMAL AND VASOSPASTIC MONKEY CEREBROVASCULAR SMOOTH MUSCLE CELLS

SUMMARY

Normal cytoarchitecture of smooth muscle cells of monkey cerebral arteries was studied using scanning electron microscopy (SEM) after removal of adventitial connective tissue by hydrolysis with HCl. Cerebral arteries were also examined after contraction *in vitro* with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Anterior cerebral arteries were studied after exposure for 6 days *in vivo* to whole blood, oxyhemoglobin (OxyHb), methemoglobin (MetHb), bilirubin, mock cerebrospinal fluid (CSF), or supernatant fluid from an incubated mixture of autologous blood and mock CSF. Normal smooth muscle cells were spindle-shaped and oriented circumferentially around the vessel. They were often grouped into bundles of 5 to 10 cells; bundles were recognizable because cells within them were joined by multiple intercellular contacts. Groups of smooth muscle cells oriented longitudinally were present outside the circular layers of cells. The adventitial surface of muscle cells was smooth apart from fine longitudinal striations in some areas. Arteries contracted with $PGF_{2\alpha}$ had markedly convoluted and folded cell membranes. Muscle cells of vasospastic arteries and of arteries exposed to OxyHb and supernatant fluid appeared identical to cells contracted with $PGF_{2\alpha}$. The outer surface of cells of arteries exposed to bilirubin, MetHb, and mock CSF were normal. Marked similarity between vasospastic smooth muscle cells and smooth muscle cells from arteries contracted with $PGF_{2\alpha}$ suggest that smooth muscle contraction occurs during vasospasm (VSP) due to whole blood and due to intrathecal injection of OxyHb.

INTRODUCTION

Cerebral VSP following subarachnoid hemorrhage (SAH) may be due to prolonged and exaggerated smooth muscle contraction.[5,11,13,14] Vasospastic arteries, however, exhibit features not typical of physiologic vasoconstriction, including impaired relaxation to pharmacologic vasodilators and stiffening of contractile and noncontractile components of the arterial wall.[2,11,24] Pathologic degeneration of smooth muscle cells in the arterial wall has also been observed.[5,13,14] To explain these findings, some authors have emphasized vessel wall fibrosis, inflammatory-cell infiltration, and cellular damage in the genesis of VSP.[2,14,24,26] Alternatively, there is evidence that the explanation for these features lies in disruption of the contractile machinery of smooth muscle.[5,11] Morphometric studies of arterial walls show that although vasospastic vessels have markedly thickened walls, they have only minor increases in vessel wall area which are not substantial enough to account for luminal narrowing (Chapter Four).[13,14]

Direct visualization of vascular smooth muscle is possible by several methods.[15] Cytoarchitecture of normal and vasospastic monkey cerebrovascular smooth muscle cells after digestion of surrounding tissues has not been reported. This study used HCl-hydrolysis treatment of fixed cerebral arteries from monkeys to study normal, pharmacologically-contracted, and vasospastic arteries as well as monkey arteries exposed *in vivo* to OxyHb, MetHb, bilirubin, mock CSF, and supernatant fluid from an incubated mixture of autologous blood and mock CSF. Objectives were to define normal cytoarchitecture of monkey cerebrovascular smooth muscle and to compare changes seen with vasoconstriction with changes of VSP.

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MATERIALS AND METHODS

Source of Specimens

A female cynomolgus monkey was killed by intravenous overdose of sodium pentobarbital and exsanguination from the heart. The circle of Willis was removed and basilar, anterior cerebral, and middle cerebral arteries (MCA) were cut into segments. Each segment was placed in 10 ml Krebs-Henseleit solution (115.0 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl, 1.2 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 10.0 mM glucose) bubbled with 95% O_2 and 5% CO_2 at 37°C in a water-jacketed tissue bath. Eight segments were contracted by adding $PGF_{2\alpha}$ to the water baths at a final concentration of 10^{-5} M. After contraction for 1 hour in this solution they were transferred to 10 ml fixative solution (3% glutaraldehyde and 1% paraformaldehyde in Millonig's buffer, 0.12 M, pH 7.4) at 4°C for up to 9 hours. Four control segments were fixed after incubation without $PGF_{2\alpha}$.

Two vasospastic MCAs from female cynomolgus monkeys were also examined. Details of angiography, exposure of basal cerebral arteries, creation of SAH, and killing of animals in our model have been described.[4] Briefly, VSP was induced by surgical placement of a 5 ml autologous blood clot in the basal cisterns. Cerebral angiography was done at baseline and immediately prior to sacrifice. Animals were killed after 7 days by pentobarbital overdose and exsanguination. Intra-arterial perfusion was performed at 110 mm Hg via a left ventricular cannula with the descending aorta cross-clamped and the right atrium opened widely. Perfusion was with 0.5 L of 0.9% saline to wash out circulating blood followed by 0.5 L fixative solution (3% glutaraldehyde and 1% paraformaldehyde in Millonig's buffer, 0.12 M, pH 7.4) at 4°C over 5 to 10 minutes.

Basilar and anterior cerebral arteries from 24 female cynomolgus monkeys were obtained from an earlier experiment (Chapter Two).[12] Following baseline angiography, the right basal arachnoid cisterns were opened and a catheter was placed along the right MCA. Intrathecal injections were given through an subcutaneous Ommaya reservoir which was connected to the catheter. Solutions injected included OxyHb, MetHb, bilirubin, mock CSF, and supernatant fluid from an incubated mixture of autologous blood and mock CSF. Angiography was repeated after 6 days of injections after which animals were killed and fixed by intra-arterial perfusion as described above. Basilar arteries served as normal controls.

Specimen Preparation

Specimens were postfixed with 2% OsO_4 for 2 hours (1 hour at 4°C, followed by 1 hour at 40°C). Arterial segments were then washed in distilled water and incubated in 8 M HCl for 20 to 40 minutes at 60°C. This procedure removes adventitial connective tissue, exposing underlying smooth muscle cells.[19] They were washed in distilled water, dehydrated in a graded series of ethanol solutions, immersed in absolute ethanol and critical-point dried (Seevac Inc., model CPD-100 CO_2 critical point dryer, Pittsburgh, Pennsylvania). Arteries were mounted on aluminum stubs, sputter coated with gold (Edwards high vacuum, model S150B, Manor Royal, Cravley, West Sussex), and examined with SEM (Model 505, N.V. Phillips Gloelampenfabrieken, Eindhoven, The Netherlands) at 5 to 30 kV.

To verify that structures observed were smooth muscle cells exposed by removal of adventitial connective tissue, several specimens were removed from SEM stubs and embedded in Araldite (Marivac Ltd., Halifax, Nova Scotia) for study by transmission electron microscopy (TEM, Hitachi H-7000, Hitachi, Tokyo, Japan).

Quantification of Morphologic Changes, Data Analysis

When changes in morphology, such as membrane folding, were observed, an estimate was made of per cent of total vessel surface area over which smooth muscle cells exhibited the change. Although it was difficult to measure lengths of smooth muscle cells, maximum diameters were measured for 30 cells from normal, vasospastic,

pharmacologically-contracted, and intrathecally-injected monkeys. For contracted smooth muscle cells, diameter at the widest point was measured. In groups where membrane folds were observed, width of 20 folds were measured. Width of 20 folds was also measured from folds seen on TEM of arteries exposed to OxyHb. All measurements were corrected by photographing a calibration grid in the microscope.

For each measurement, values are expressed as means ± standard deviation. Values were compared to measurements for control cells by unpaired t-test for cell diameters. Analysis of variance followed by Scheffe's multiple comparison procedure was used to compare values for width of folds. $P < 0.05$ was taken as significant.

RESULTS

The HCl-digestion removed adventitial connective tissue from cerebral arteries, exposing smooth muscle cells of the tunica media. Arterial segments reembedded for TEM after observation by SEM confirmed that structures observed were smooth muscle cells and that most extracellular matrix within tunica media had also been removed by HCl treatment (Fig. V-1).

Normal Anatomy

Views of cut ends of these vessels showed they were relaxed as evidenced by minimal pleating of the internal elastic lamina and the flat endothelial cell layer. Smooth muscle cells were arranged circumferentially, perpendicular to the long axis of the artery (Fig. V-2). Muscle cells were often grouped into bundles of 5 to 10 cells. These cell bundles were interconnected by numerous cell-cell contacts (Fig. V-3). Occasionally, bundles had a slightly helical orientation around the vessel (Fig. V-2). Bundles of the outermost layer of muscle cells sometimes intruded between adjacent groups of cells and merged with cells deeper in the media (Fig. V-2).

Cells were tube-shaped with uniform diameters, except at their tapering ends (Fig. V-2). Outer surfaces of cells were generally smooth. Mild folding of the adventitial surface of smooth muscle cells was present over less than 10% of vessel surface area. Fine longitudinal striations of the cell surface were also noted but these were not common (Fig. V-4).

Longitudinal bundles of smooth muscle cells were observed on the outer aspect of the tunica media (Fig. V-2). These were observed at branch points where the artery divided or gave off a small tributary (Fig. V-4) as well as along otherwise straight sections of artery. They were observed in only about 10% of segments examined. Average diameter of normal smooth muscle cells was 2.8 ± 0.8 μm (Table V-1).

$PGF_{2\alpha}$-Contracted Arteries

Contraction of monkey cerebral arteries with $PGF_{2\alpha}$ produced marked changes in morphology of smooth muscle cells which were observed over 50% of surface area of all segments examined (Fig. V-5). Cells assumed a beaded appearance with narrow segments alternating with bulbous widenings. Over widenings, the cell membrane was thrown into numerous ridges, folds, and nodular projections which generally ran transversely across the cells. Width of these folds was 0.32 ± 0.12 μm (Table V-1). There appeared to be flattening of some cells along the longitudinal axis of the vessel with most cell membrane pleating occurring on the abluminal aspect of the cell. Observation of cut ends of arteries also showed folding was most prominent on the adventitial surface of smooth muscle cells but that there was some corrugation of cell membranes of cells deeper in the media. Inner-most smooth muscle cells were thrown into violent folds to conform to the pleated internal elastic lamina. Surfaces of these cells were covered in fine folds as well. Diameter of cells contracted with $PGF_{2\alpha}$ was 3.4 ± 0.7 μm which was significantly greater than diameter of normal cells ($p < 0.005$, Table V-1).

Vasospastic Arteries

Monkey MCAs exposed to subarachnoid blood clot for 7 days and with angiographically-confirmed VSP showed changes similar to those observed in vessels treated with $PGF_{2\alpha}$ (Fig. V-5). Smooth muscle cells assumed a beaded configuration with widened areas interposed between narrow areas. Corrugation and folding of the cell membrane was present over widened areas. Smooth muscle over approximately 70% of vessel surface area exhibited these changes in all segments examined. Diameter of these cells was 3.4 ± 0.9 μm and width of folds was 0.31 ± 0.12 μm (Table V-1). Fiber diameter was significantly greater than diameter of normal muscle fibers ($p < 0.01$).

Study of anterior cerebral arteries from monkeys given intrathecal injections of OxyHb, MetHb, bilirubin, mock CSF, and supernatant fluid gave results which corresponded to results of angiography on those monkeys. Significant VSP occurred in animals injected with OxyHb and supernatant fluid.[12] Bilirubin caused slight but insignificant VSP while MetHb and mock CSF were inert. Accordion-like pleating of muscle cell membranes was present in arteries from monkeys injected with OxyHb and supernatant fluid (Fig. V-6). This occurred over 30% to 40% of surface area of each segment examined. Arteries from other groups were normal. Diameters and width of folds for muscle cells in these groups are shown in Table V-1. Similar to contracted or vasospastic smooth muscle, muscle cells from arteries exposed to OxyHb and supernatant fluid had increased diameters compared to normal fibers, although these increases were significant only after injection of OxyHb ($p < 0.05$). Width of folds seen on cells exposed to OxyHb and supernatant fluid were not significantly different in size to those seen in other groups (Table V-1). On TEM of MCAs from monkeys with VSP due to OxyHb or supernatant fluid, plication of the smooth muscle cell membrane was seen, corresponding to changes observed by SEM (Fig. V-1 and Table V-1). Dense bodies were often observed adjacent to the cell membrane between the folds. The size of folds was comparable to folds seen on cells contracted by whole blood, OxyHb, supernatant fluid, and $PGF_{2\alpha}$ (Table V-1).

DISCUSSION

Specimen Preparation

The precise arrangement and morphology of vascular smooth muscle has proven difficult to study using two-dimensional thin sections and conventional microscopy techniques. Several methods have been developed whereby arterial adventitia can be removed, leaving smooth muscle cells of tunica media exposed and undamaged.[16] These include blunt dissection, microdissection, and chemical digestion, the latter most commonly done with HCl and collagenase. Digestion with chemicals has produced good results and cytoarchitecture of blood vessels in many systemic organs has been reported.[18]

Anatomy of Normal and Contracted Smooth Muscle

This study describes normal anatomy of smooth muscle cells of cynomolgus monkey cerebral arteries. Shiraishi et al [19] treated dog cerebral arteries by HCl-hydrolysis. Arrangement and morphology of smooth muscle cells which they describe in dogs appears identical to cynomolgus monkeys. The majority of muscle cells are oriented perpendicular, not oblique, to the long axis of the artery. With vasoconstriction, there appears to be some tendency for cells to develop a helical orientation. Contraction *in vitro*, however, allows the artery to shorten and thus, firm statements regarding orientation of cells are not possible. Others have reported that during constriction, cells remain circumferentially-oriented.[28]

Shiraishi and colleagues [19] also describe longitudinally-oriented bundles of muscle cells at the adventitia-media border of dog cerebral arteries. These were observed at branching and non-branching points in monkey arteries studied at our center. Longitudinal bundles were not found in a study of rat cerebral arteries.[22]

Function of these cells is unclear although they may help prevent excessive longitudinal stress.

The adventitial surface of relaxed vascular smooth muscle is smooth with only mild and focal pleating of the cell membrane.[15,22] Fujiwara and Uehara found extensive folding and nodularity of the outer surface of smooth muscle cells of monkey mesenteric arteries.[6] It was later reported that these arteries were not completely relaxed and that the folded appearance of smooth muscle was due to contraction.[15] Further studies have demonstrated that relaxed muscle cells have a smooth adventitial surface and constriction causes folding and corrugation of the cell membrane, similar to changes observed in $PGF_{2\alpha}$-treated and vasospastic arteries of this study.[15,16,21] In the report by Uehara and Fujiwara, monkey mesenteric arteries developed more coarse projections and nodules on their outer surface, as opposed to fine pleating seen in cerebral arteries of this study. This could be due to different constraints put on the outer surface of the cell by presence of an external elastic lamina (which is perforated by multiple pores [6]) and thicker adventitia of systemic arteries. It might also be due to variation in configuration of the contractile apparatus in the cells. Blebs but not fine folds, similar to nodules described above, were observed on isolated smooth muscle cells contracted *in vitro* by electric stimulation.[3]

Cell membrane folding in contracted smooth muscle cells was observed as early as 1969 when Phelps and Luft used TEM to study frog arterioles constricted with epinephrine.[17] They noted indentation and convolution of smooth muscle cell membranes in arteries exposed to epinephrine. Review of other publications also shows this corrugation occurs during muscle contraction.[1,7] We also observed these changes in TEM of vasospastic vessels (Fig. V-1). Sizes of folds seen on SEM were comparable to those observed on TEM (Table V-1). Two-dimensional microscopy, however, did not allow elucidation of orientation of folds with respect to the axis of muscle cells.

The cause of cell membrane folding during muscle contraction is unclear. Gabella pointed out that shortening of a tube-shaped cell would produce excess membrane material if diameter of the tube remained constant.[7] Extra membrane would then be thrown into folds, the configuration of which would depend on the collagen surrounding the cell. It is known that subplasmalemmal dense bodies of smooth muscle are anchoring points of actin filaments. Fay and Delisle proposed that during contraction, folds of membrane were thrown out between dense bodies in order to accomodate cell cytoplasm displaced by cell shortening (Fig. V-7).[5] On TEM, we often observed dense bodies between the folds of membrane, as others have reported.[7]

Fine longitudinal striations on the surface of smooth muscle cells observed in this study were also described by Shiraishi et al.[19] They may be formed by the configuration of dense bodies within the cell. Cell junctions noted in this study have been well characterized by TEM and are predominately gap junctions.[9]

Vasospasm

This study shows that smooth muscle cells of vasospastic monkey MCAs have features which are identical to those of arteries vasoconstricted *in vitro* with $PGF_{2\alpha}$. OxyHb also causes VSP in monkeys [12] and produces morphologic changes in cerebrovascular smooth muscle which are consistent with smooth muscle contraction. Although the contribution of smooth muscle contraction to arterial narrowing of VSP has been disputed,[2] this study shows that even after 7 days, contraction persists in cerebral arteries exposed to whole blood and to OxyHb. Since the contribution to VSP of pathologic changes which increase arterial wall mass is probably not significant,[14] lumen narrowing of VSP must be due, at least initially, to smooth muscle contraction. Explanations for why tachyphylaxis does not develop to contraction induced by OxyHb, why vasospastic arteries fail to relax in response to vasodilator drugs,[2] and why vasospastic arteries have decreased compliance [11,24] may lie in disruption of the cellular contractile apparatus caused by prolonged release of high concentrations of OxyHb into CSF following SAH. The similar size of folds in pharmacologically-contracted and vasospastic smooth muscle suggests that contraction is mediated in both cases by a

similar mechanism. There is, however, no consensus about what intracellular processes mediate tonic smooth muscle contraction.[8,10,18] Few investigations into intracellular processes occurring in smooth muscle during VSP have been published.[20,23] One study found VSP in dogs did not change levels of enzymes involved in phasic smooth muscle contraction (myosin light chain kinase, cyclic adenosine monophosphate-dependent protein kinase, and cyclic guanosine monophosphate-dependent protein kinase) in basilar artery.[20] There is evidence, however, that OxyHb increases smooth muscle inositol trisphosphate, a known intracellular second messenger which mediates intracellular Ca^{2+} release.[23]

The technique used in this study did not allow assessment of other factors which might fix vasospastic arteries in a contracted state for prolonged periods. Acid hydrolysis destroys extracellular collagen and connective tissue. Increases in extracellular collagen, which might be involved in holding arteries in a contracted state,[2,24,26] would therefore not be demostrated using this techique. Proliferation of contractile myointimal cells could also contribute to VSP.[26] These cells, if present, would not be readily observed in this study since observations were confined mainly to the outer surface of the tunica media.

Table V-1: Diameters of Smooth Muscle Cells and Width of Folds on Smooth Muscle Cells Observed After Acid Hydrolysis of Arterial Segments, by Group. Values are Means ± Standard Deviations (n = 30 in each group for diameters and n = 20 for fold widths in each group).

Group	Diameter (μm)	Fold Width (μm)
Control	2.8 ± 0.8	-
Prostaglandin $F_{2\alpha}$-contracted	3.4 ± 0.7 [a]	0.32 ± 0.12
Whole Blood	3.4 ± 0.9 [a]	0.31 ± 0.12
Oxyhemoglobin	3.2 ± 0.6 [a]	0.32 ± 0.09
Methemoglobin	2.8 ± 0.6	-
Bilirubin	2.8 ± 0.7	-
Supernatant Fluid	3.2 ± 0.7	0.33 ± 0.08
TEM of Oxyhemoglobin	-	0.34 ± 0.11

TEM, transmission electron micrographs.

[a] significantly different from control value ($p < 0.05$, unpaired t-test).

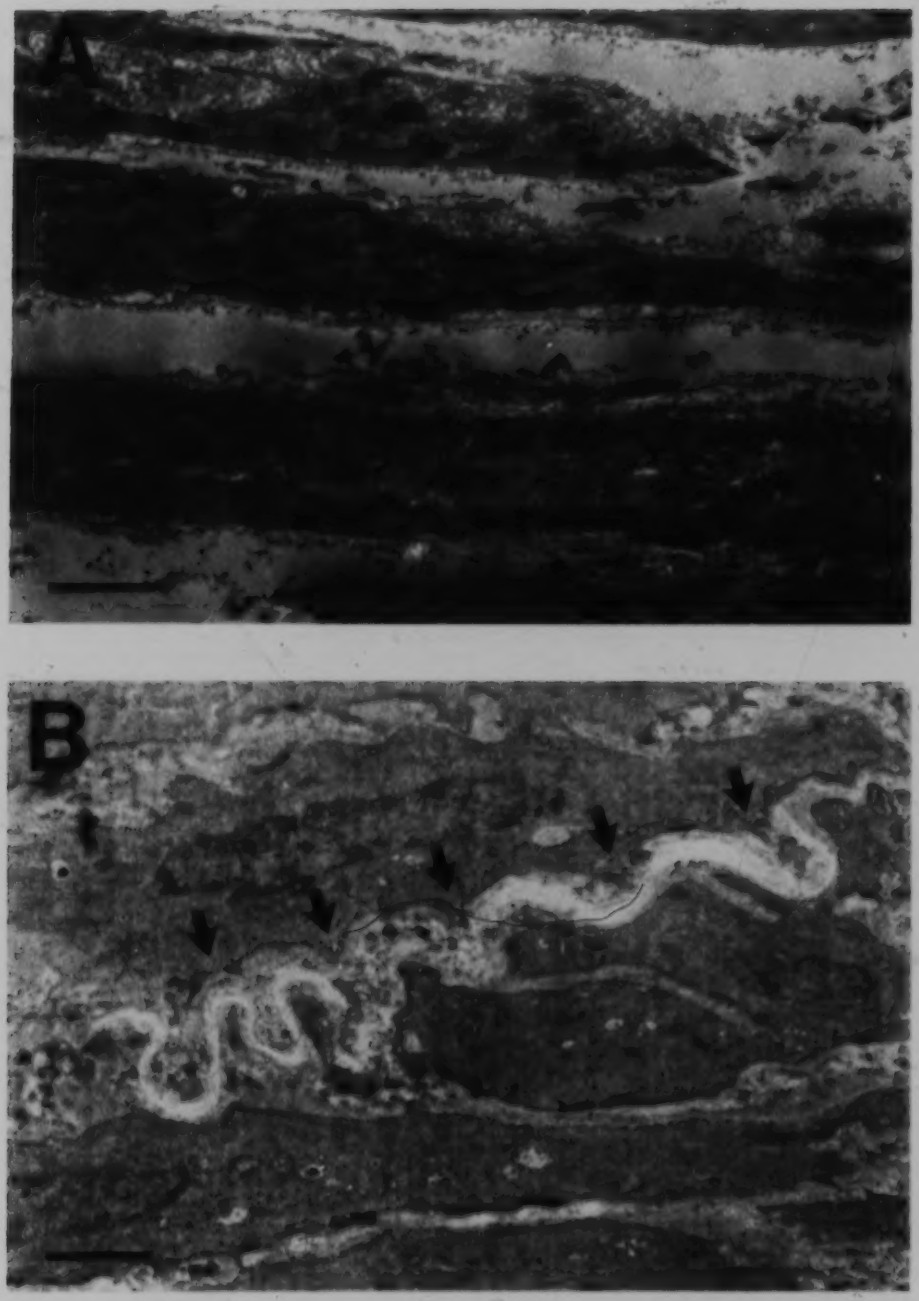

Figure V-1: A: transmission electron photomicrograph showing appearance of normal middle cerebral artery reembedded and studied by transmission electron microscopy after acid hydrolysis treatment and examination by scanning electron microscopy (75 kV). Most extracellular and adventitial connective tissue is absent. Scale bar = 2 μm. B: transmission electron photomicrograph showing folded cell membrane (arrows) of smooth muscle cells in the tunica media of a middle cerebral artery from a monkey given intrathecal injections of oxyhemoglobin (75 kV). Scale bar = 2 μm.

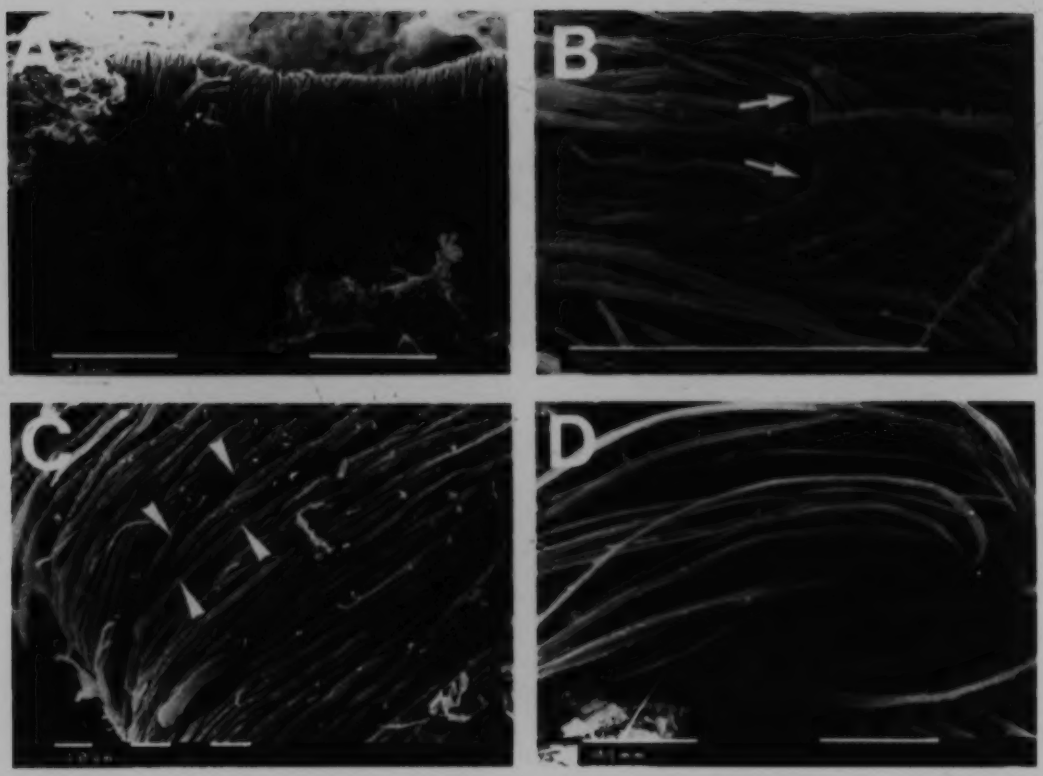

Figure V-2: Scanning electron photomicrographs of normal anatomy of monkey cerebral arterial smooth muscle cells. Scale bars are present below each photomicrograph. A. Segment of normal anterior cerebral artery showing that smooth muscle cells are oriented circumferentially with some bundles having a slightly helical orientation. Fragments of adventitia are seen adherent to the medial smooth muscle cells (asterisks)(15 kV). B. Normal basilar artery segment showing a group of longitudinal smooth muscle cells located at the media-adventitia border (arrows)(15 kV). C. Normal basilar artery showing circumferential muscle cell bundles with one group of cells interdigitating between other cells and merging with cells deeper in the media (arrowheads)(15 kV). D. Individual smooth muscle cells from a normal basilar artery. Cells have a fine tubular shape with smooth cell membranes (25 kV).

Figure V-3: Scanning electron photomicrograph of anterior cerebral artery from a monkey given intrathecal injections of oxyhemoglobin. There are multiple intercellular contacts visible between smooth muscle cells (arrows). Mild folding of the cell membrane is seen (30 kV, scale bar = 10 µm).

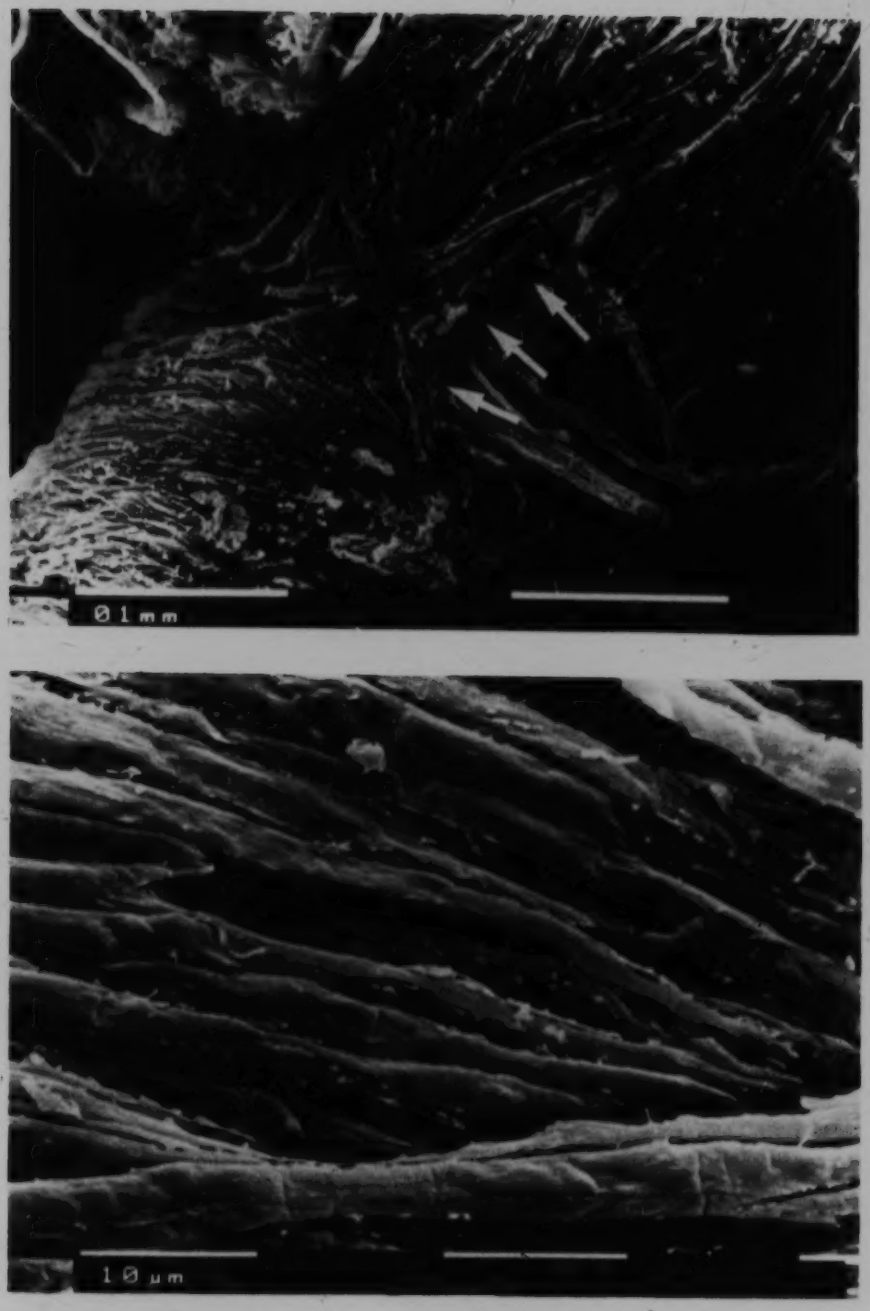

Figure V-4: Top: scanning electron photomicrograph of branch point of two cerebral arteries showing a transversely-oriented group of muscle cells spanning the junction (arrows)(25 kV). Bottom: fine longitudinal striations are present on surfaces of smooth muscle cells of a basilar artery from an animal given intrathecal injections of bilirubin (15 kV). Scale bars are present below each photomicrograph.

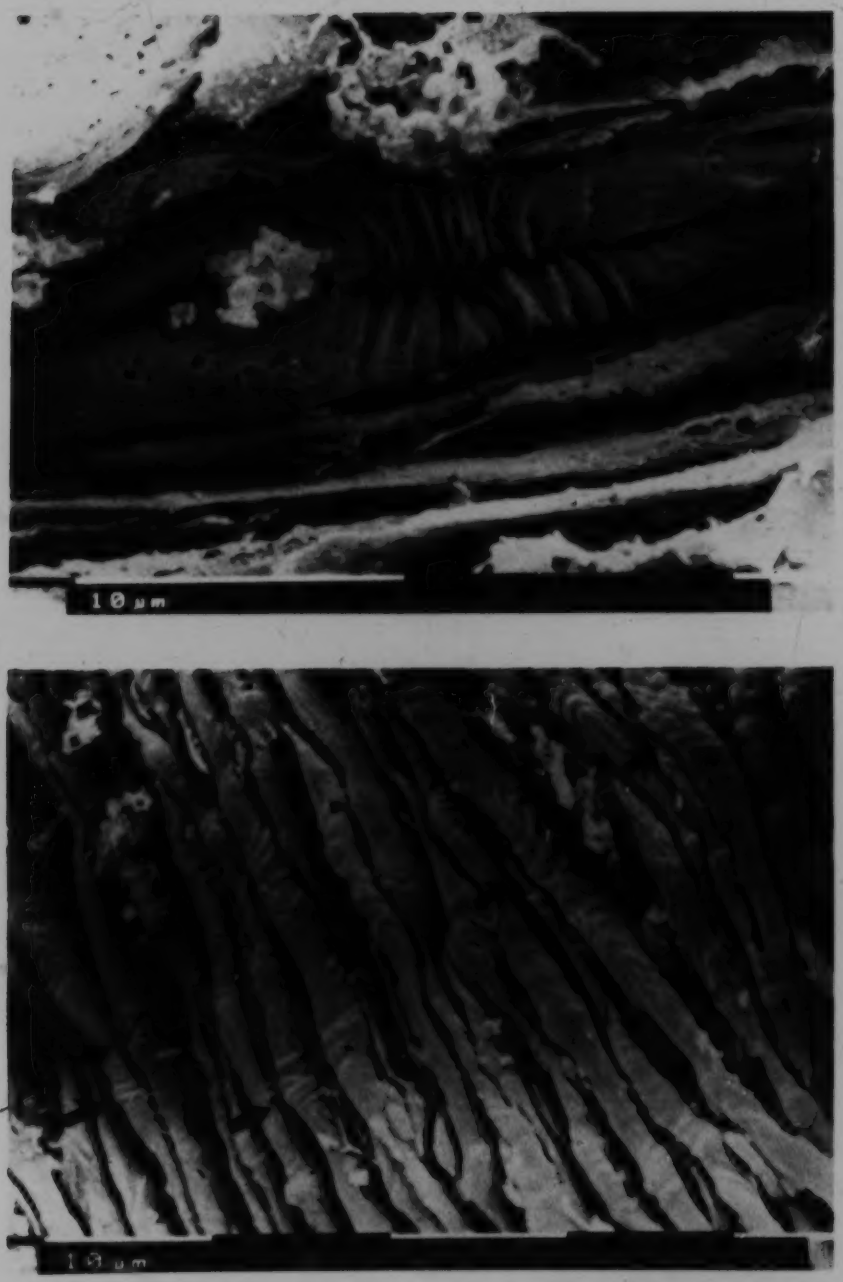

Figure V-5: Scanning electron photomicrographs of monkey basilar artery contracted *in vitro* with prostaglandin $F_{2\alpha}$ (top) and of monkey middle cerebral artery made vasospastic by exposure *in vivo* for 7 days to subarachnoid blood clot (bottom). Note the similar appearance with marked folding of the cell membranes (arrows) of smooth muscle cells generally occurring over widened areas of the cells (5 kV [top] and 15 kV [bottom]). Scale bars are present below each photomicrograph.

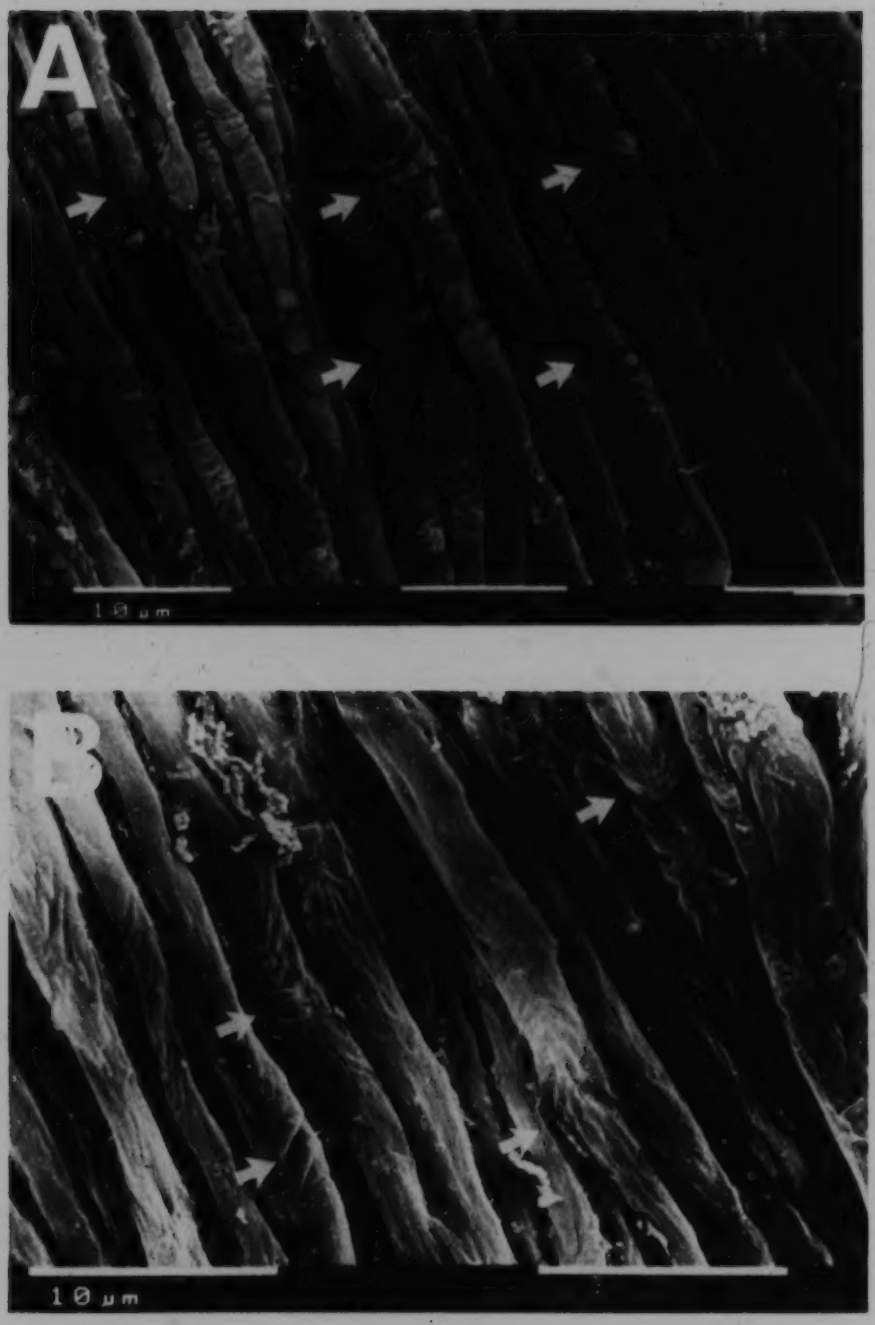

Figure V-6: Scanning electron photomicrographs of monkey anterior cerebral arteries with vasospasm due to intrathecal injections of supernatant fluid (A) and oxyhemoglobin (B). Rippling of the cell membrane of muscle cells (arrows) is seen after exposure to both of these substances (15 kV [top] and 30 kV [bottom]). Scale bars are present on each photomicrograph.

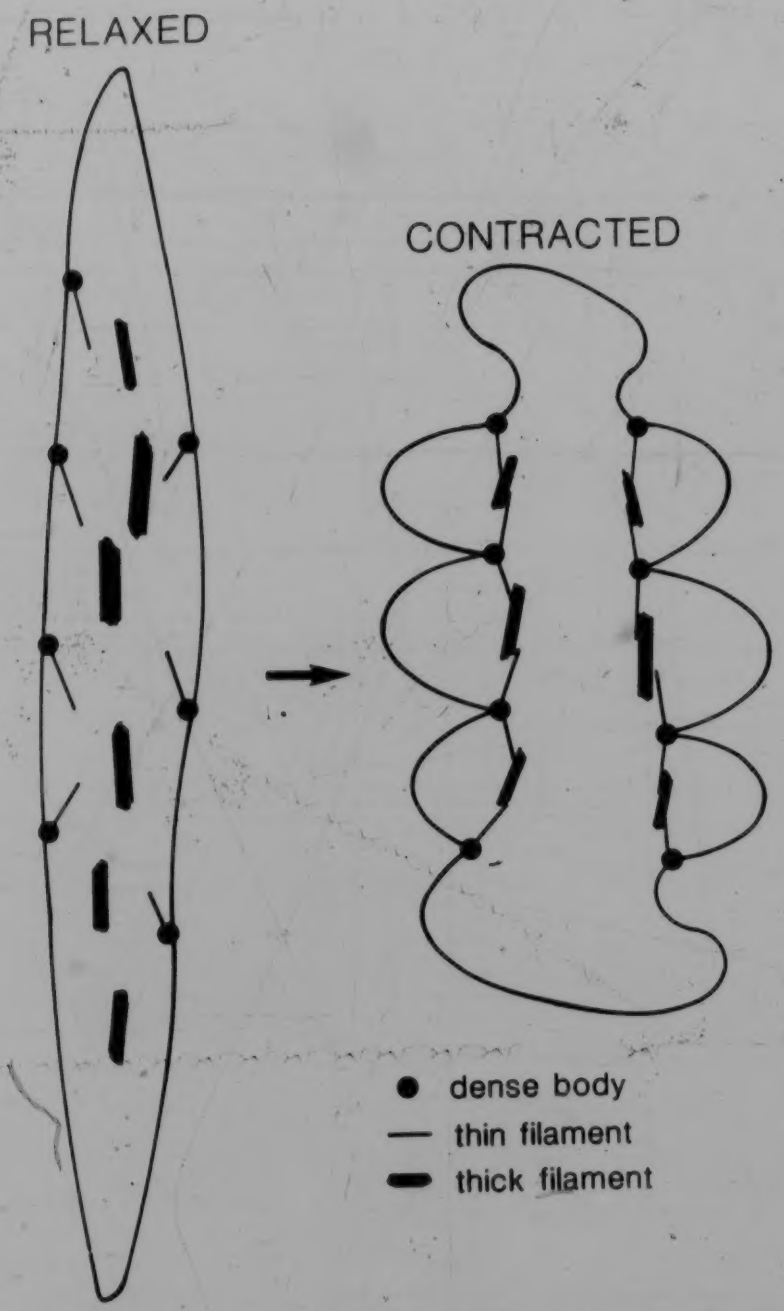

Figure V-7: Diagram demonstrating a possible mechanism for genesis of cell membrane folding during smooth muscle contraction.[3] During contraction, thin (actin) and thick (myosin) filaments interact and slide past one another, throwing excess membrane material out between dense bodies to which thin filaments are attached. In reality, there could be multiple thin and thick filaments spanning the interval between membrane folds.

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CHAPTER SIX [a]
CYTOSKELETAL AND EXTRACELLULAR MATRIX PROTEINS IN CEREBRAL ARTERIES FOLLOWING SUBARACHNOID HEMORRHAGE IN MONKEYS

SUMMARY

It is unclear if vasospasm (VSP) after subarachnoid hemorrhage (SAH) is predominately due to smooth muscle contraction, proliferative vasculopathy, or other changes within the arterial wall such as fibrosis or change in smooth muscle phenotype. Immunohistochemistry was used to examine changes in extracellular and cytoskeletal proteins in cerebral arteries after SAH which might support one of these mechanisms. Following baseline cerebral angiography, bilateral SAH was created in 9 monkeys. Animals were killed after 7 (n = 3), 14 (n = 3), and 28 (n = 3) days. Cerebral angiography was repeated on day 7 in all animals and immediately prior to animals being killed on days 14 and 28. Both middle cerebral arteries (MCA) from each animal and 4 control basilar arteries (BA) were examined using fluorescent antibody techniques with antisera to α-actin, myosin, fibronectin, fibrinogen, vimentin, desmin, laminin and collagens (types I, III, IV, and V). Arteries were also stained using Verhoeff's elastic stain and by immunoperoxidase method for factor 8-related antigen. Angiography showed VSP was most severe on day 7, that it was present but resolving on day 14 and that vessels were normal angiographically on day 28. Study of sections at microscopy and blinded review of photographs of arterial sections by 5 independent observers did not reveal changes in intensity or density of staining for the different collagens, desmin, myosin, laminin, or α-actin in tunica media or tunica adventitia. Fibronectin immunoreactivity increased 14 days after SAH. Seven days after SAH, occasional areas of tunica media showed immunoreactivity to fibrinogen. On day 28, intimal thickening was observed in 4 of 6 middle cerebral arteries and this tissue contained immunoreactivity to α-actin, myosin, vimentin, desmin, fibronectin, laminin, and each type of collagen. Significant increases in intimal cells showing immunoreactivity to α-actin did not occur at any time after SAH. No changes in staining for elastic tissue or factor 8-related antigen were noted. No significant changes in hydroxyproline content of cerebral arteries developed at any time after SAH. These results suggest rigidity and lumen narrowing of VSP is not due to increased arterial collagen, although changes in other unidentified arterial wall components or cross-linking of extracellular matrix constituents could account for these changes. There is no indication that smooth muscle contractile proteins change during VSP or that increases in α-actin-containing myointimal cells contribute to VSP. The occurrence of intimal thickening and increased tunica media fibronectin after VSP suggests VSP damages smooth muscle, possibly by producing intense, prolonged, and nonphysiologic vasoconstriction.

INTRODUCTION

The mechanism by which subarachnoid clot causes VSP after aneurysmal SAH is controversial. Vasospasm could be primarily due to smooth muscle contraction which is prolonged and temporarily irreversible secondary to its mediation by high and continuous concentrations of, for example, oxyhemoglobin.[15] Electron microscopy confirms that smooth muscle is contracted during VSP.[12,16,37] There is, however, evidence that processes occur in arterial walls during VSP which are not consistent with vasoconstriction. Mayberg et al found that VSP in piglets and in femoral arteries of rats was associated with decreases in immunoreactivity to actin.[29,36] Pharmacologic

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studies generally show vasospastic arteries have decreased contractility, increased spontaneous tone, and increased stiffness compared to normal cerebral arteries.[2,3,6,23,25,48] These findings, in conjunction with reports of increased tunica media collagen [6,15,29,46,48] and arterial wall inflammation [8,15,20,29] in vasospastic arteries, raise the possibility that SAH causes primary vessel wall damage and that these changes are more important than vascular smooth muscle contraction in generating and maintaining VSP. Bevan et al proposed that VSP begins as smooth muscle contraction but that other processes, including inflammation and collagen deposition, are responsible for chronic narrowing.[2,3,48]

A third theory invokes proliferation of cells and connective tissue within vasospastic arterial walls, resulting in increased wall mass which then contributes primarily to lumen narrowing.[6,24] The timing of such changes, however, remains controversial [20] and measurements of vessel wall areas after SAH show that increases in wall mass are of insufficient magnitude to encroach on the lumen.[29]

We have used immunohistochemistry and amino acid analysis to study cerebral arteries from monkeys during development and resolution of VSP after SAH. The purpose was to define the time course of changes in arterial wall collagens, myointimal cells, and in smooth muscle contractile and intermediate filament proteins after SAH and, therefore, to determine which changes could be responsible for narrowing and stiffening of vasospastic arteries.

MATERIALS AND METHODS

Protocol

Nine female monkeys (Macaca fascicularis) weighing between 2.93 and 4.70 kg were divided by restricted randomization into 3 equal groups to be killed 7, 14, and 28 days after SAH (Fig. VI-1). Baseline physical assessment and cerebral angiography, induction of subarachnoid hemorrhage, killing, and radiologic analysis were carried out as previously reported.[14] On day 0, all animals underwent baseline cerebral angiography and bilateral subarachnoid clot placement. Angiography was repeated 7 days later in all animals. Animals killed on days 14 and 28 also had angiography repeated immediately prior to termination. Monkeys were killed and fixed by perfusion after which both MCAs and BAs were removed for pathologic examination.

All protocols were approved by the Animal Ethics Review Committee of the University of Alberta. Guidelines of the Canadian Council on Animal Care for care and surgery of monkeys were followed.

Perfusion-Fixation

Sodium pentobarbital (50 mg/kg) was administered intravenously after completion of the final angiogram. A left ventricular cannula was inserted after cross-clamping the descending aorta and opening the right atrium. Perfusion was at 110 mm Hg with 0.5 L 0.9% NaCl followed by 0.5 L modified Bouin's solution (15% picric acid, 2% paraformaldehyde, and 5% sucrose in 0.2 M phosphate buffer, pH 7.6) at 4°C for 10 minutes. Brains and residual clot were removed and photographed. The entire circle of Willis was dissected from the brain and placed in modified Bouin's solution for 4 hours at 4°C. Arteries were then washed several times in phosphate buffer (12% sucrose in 0.2 M sodium phosphate buffer, pH 7.6) and stored at 4°C in this buffer until sectioning.

Tissue Processing

Segments of MCA and BA were embedded in OCT compound (Tissue-Tek, Miles Pharmaceuticals, Ekhart, Indiana) and rapidly frozen on dry ice. Cross-sections 10 μm thick were cut in a cryostat (Reichert-Jung Frigocut model 2800, West Germany) and mounted on glass slides coated with aminopropyltriethoxy-silane (Sigma Chemical Co., St. Louis, Missouri).[27] All sections were permeabilized with Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered saline (10 mM Hepes, 150

mM NaCl, pH 7.4) containing 0.1% triton X-100 and then blocked for 20 minutes with Hepes-buffered saline containing Ca^{2+}, Mg^{2+} (10 mM Hepes, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4), and 2% fetal calf serum. Slides were exposed to the following primary antibodies under the described conditions: rabbit polyclonal anti-desmin antisera (Chemicon International, Inc., Temecula, California), diluted 1/40, 9 hours at 4°C, rabbit polyclonal anti-human type I collagen antisera (Chemicon International, Inc., Temecula, California), diluted 1/40, 9 hours at 4°C, rabbit polyclonal anti-human type III collagen antisera (Chemicon International, Inc., Temecula, California), diluted 1/20, 16 hours at 4°C, rabbit polyclonal anti-human type IV collagen antisera (Chemicon International, Inc., Temecula, California), diluted 1/320, 9 hours at 4°C, goat polyclonal anti-human type V collagen antisera (Southern Biotechnology Associates, Inc., Birmingham, Alabama), diluted 1/200, 16 hours at 4°C, mouse monoclonal anti-vimentin antibody (Sigma Chemical Co., St. Louis, Missouri), diluted 1/100, 16 hours at 4°C, mouse monoclonal anti-α-actin antibody (Boehringer Mannheim Canada, Ltd., Laval, Quebec), diluted 1/50, 2 hours at room temperature, mouse monoclonal anti-fibrinogen antibody (AMAC, Inc., Westbrook, Maine), diluted 1/50, 18 hours at 4°C, mouse monoclonal anti-fibronectin antibody (Biodesign International, Kennebunkport, Maine) diluted 1/50, 22 hours at 4°C, rabbit polyclonal anti-laminin antisera (Gibco Laboratories, Inc., Grand Island, New York), diluted 1/100, 18 hours at 4°C, and mouse monoclonal anti-myosin antibody (AMAC, Inc., Westbrook, Maine), diluted 1/20, 16 hours at 4°C. After incubation with primary antisera, slides were washed in Hepes-buffered saline containing Ca^{2+} and Mg^{2+} and incubated for 1 hour at room temperature with biotinylated secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, Pennsylvania) diluted 1/1000 and directed against the species and immunoglobin type of the primary antibody. After washing again, Texas-red conjugated with streptavidin (Amersham Canada, Ltd., Oakville, Ontario) was added for 30 minutes at room temperature. Slides for α-actin were also reacted with fluorescein isothiocyanate (FITC)-labelled secondary antibodies after application of the primary mouse α-actin antibody. Slides were washed twice with buffer, mounted in a solution of glycerol and mowiol, and viewed under a Zeiss Axiovert 35 fluorescence microscope (Carl Ziess, Inc., West Germany) equipped with epifluorescence optics, a 40X plan neofluar oil immersion lens (numerical aperature 1.30), and filters for detection of FITC and Texas red. All specimens were photographed at constant magnification (X 40) and exposure time with Kodak T/MAX 400 ASA black and white film or Kodak extachrome 400 ASA color slide film (Kodak Canada, Toronto, Ontario).

Sections were stained for elastic tissue using Verhoeff's elastic stain. For detection of factor 8-related antigen, immunoperoxidase methods were used. Slides were stained with hematoxylin and rinsed in distilled water, blocked with 20% horse serum for 10 minutes at 37°C, and incubated for 30 minutes at 37°C with mouse monoclonal anti-factor 8-related antigen antibody (Dakopatts A/S, Glostrup, Denmark) diluted 1/50. They were washed in 3 changes of phosphate buffered saline (1 mM, pH 7.4) and incubated for 20 minutes at 37°C with biotinylated anti-mouse immunoglobulin diluted 1/200. This was followed by successive incubations in hydrogen peroxide (10% in absolute methanol) for 2 minutes, avidin-biotin complex (Vector Laboratories, Inc., Burlingame, California) for 40 minutes, and a saturated solution of 3,3'-diaminobenzidine (8 mg/dL with 0.6% hydrogen peroxide in 1 mM phosphate buffered saline, pH 7.4). They were coverslipped wth xylene and viewed under a Zeiss photomicroscope (Carl Ziess, West Germany) equipped with plan-neofluar 16X and 25X objectives, and photographed as described above.

Sections were also processed with first antibody omitted and with rabbit, goat, and mouse immunoglobins as first antibodies used at the same concentrations as primary antibodies. Collagen antibodies used in this study are commercially available and have been shown by enzyme-linked immunosorbent assay to have minimal cross-reactivity with other collagen types and with fibronectin and laminin. Similarly, laminin antisera did not cross react with fibronectin or type IV collagen. The

fibronectin antibody did not cross react with collagens. The α-actin antibody was previously shown to be specific for the α-actin isoform of actin.[44] In addition, we incubated 1 ml volumes of types I, III, and V collagen antibodies in the concentrations used, for 24 hours at 4°C with: 1. 776 μg type I collagen, 2. 776 μg type III collagen, 3. 167 μg type IV collagen, 4. 155 μg type V collagen, 5. 50 μg laminin, or 6. 130 μg fibronectin (each obtained from Sigma Chemical Co., St. Louis, Missouri). Solutions were centrifuged at 12,000 X g for 10 minutes and used for staining.

For slides incubated with anti-α-actin antibodies, cells in the tunica intima which showed immunoreactivity to α-actin were counted in 4 sections from each artery.

Measurement of Hydroxyproline

Segments of right and left MCA for each animal were combined and analyzed along with 4 control BA. Samples were dryed and defatted by sequential 24 hour extractions in acetone, chloroform/methanol (2:1 v/v), chloroform/methanol (3:1 v/v), and ether. After weighing, samples were placed in prefired borosilicate glass tubes and sealed under N_2 vacuum. They were subjected to acid hydrolysis in 400 μL 6 N HCl containing 0.1% phenol in the sealed, evacuated glass tubes at 110°C for 22 hours.[11] They were dried under vacuum in a dessicator containing NaOH granules and resuspended in buffer and analyzed for content of amino acids, including hydroxyproline, on a Beckman model 6300 high-performance amino acid analyzer (Beckman Instruments Inc., Palo Alto, California) according to a standard protocol provided by the manufacturer.

Assessment of Immunofluorescence, Data Analysis

Intergroup comparisons of physiologic parameters (body weight, blood pressure, heart rate, end-tidal partial pressure of CO_2, and inspired partial pressure of O_2) and angiographic vessel diameters were by paired t-test. For angiographic vessel diameters, measurements for right and left sided vessels were pooled. Comparison between groups was made by analysis of variance.

In addition to review of sections by microscopy, photographs of arteries incubated with the various antibodies were reviewed by 5 independent observers without knowledge of antigens stained for or of what time period photographs were from. Intensity and density of staining in both tunica adventitia and tunica media were graded on a 3-point scale. Interobserver variability was calculated for each stain by Kendall's coefficient of concordance. Friedman's test was used to assess differences in staining for each antigen within groups.

Number of cells in tunica intima staining positive for α-actin for each artery was divided by arterial circumference at baseline to correct for differences in diameter between basilar artery and MCA. Means were obtained for each time period. Comparisons were made between each group and control BA by unpaired t-test.

Mean hydroxyproline content of arteries for each time period was calculated and expressed per mol of leucine, per mg of dried defatted weight, and per mg of protein. Protein content of samples was estimated by assuming an average residue weight of 100.[11] Ten μmol total amino acids in a sample then equals 1 mg total protein. Degree of proline hydroxylation was estimated by the ratio of hydroxyproline to proline for each time after SAH.[36] Numbers of residues of each type of amino acid per 1000 residues for each time period after SAH were also calculated. Intergroup comparisons with BA were made by paired t-test. Unless otherwise stated, all values are means ± standard deviations. P values less than 0.05 were taken to represent significant differences.

RESULTS

There were no significant differences between groups in measured physiologic values at baseline (Table VI-1). Between groups on day 7, animals in the day 28 group had significantly higher PO_2 compared to animals killed on day 7 and day 14. Comparisons within groups over different time periods showed no significant changes in physiologic parameters.

Clinical Status, Angiographic Vasospasm

One monkey in the day 14 group died after baseline angiography and was not replaced. All other animals remained well and no focal neurologic deficits were noted. Comparisons of angiograms taken on day 0 and on day 7 revealed significant reduction in MCA diameter for animals killed 7 days after SAH (50% ± 16%, $p < 0.001$). Comparison of angiograms taken at baseline and 7 days after SAH showed significant MCA VSP in animals later killed on day 14 (55% ± 8%, $p < 0.001$) and on day 28 (54% ± 4%, $p < 0.001$)(Tables VI-1 and VI-2, Figs. VI-2 to VI-5). In animals killed on day 14, comparison of day 0 and day 14 angiography showed significant MCA narrowing persisted (16% ± 10%, $p < 0.05$). Animals killed on day 28 had no significant MCA VSP as judged by comparison of day 28 angiograms with day 0 angiograms.

Immunofluorescence Microscopy

Omission of the first antibody or use, instead, of preimmune rabbit, mouse, or goat immunoglobulin resulted in no additional staining above the small amount of normal tissue autofluorescence (Fig. VI-6). Selective inhibition of staining with types I, III, and V collagen antibodies was observed when they were incubated with types I, III, and V collagen, respectively (Fig. VI-7). Incubation with other antigens did not alter staining pattern or intensity.

Results of blinded review of photographs of sections showed there was significant agreement between observers for every antigen stained ($p < 0.05$, Table VI-3). Observers were in agreement over 90% of the time amongst all 5 raters.

Extracellular Matrix Proteins: Similar patterns of immunoreactivity were seen for interstitial collagens (types I and III)(Figs. VI-8 and VI-9). Fluorescence was most intense in tunica adventitia where large fibrils fluoresced. Diffuse fluorescence was also observed in tunica media where fibrils could be demonstrated coursing through the media. In normal tunica intima, immunoreactivity to type III collagen was detected and appeared to be in the extracellular space. Only very minimal type I collagen was present in normal intima. There were no changes in intensity or density in these collagens in tunica media or adventitia at any time period following SAH (Table VI-3). In 2 of 6 MCAs from monkeys killed on day 28, tunica intima was normal. Four MCAs showed intimal thickening over 25% to 50% of the arterial circumference. Circumferential intimal thickening was not seen and thickening did not encroach significantly on the lumen, at least in the intradural arteries (Table VI-2). Both type I and type III collagen, however, were deposited in thickened intima of day 28 MCAs.

For type IV collagen, a fine reticular pattern of fluorescence surrounded smooth muscle cells in tunica media (Fig. VI-10). In tunica intima, type IV collagen was observed around myointimal cells and beneath endothelial cells. Occasional areas of type IV collagen immunoreactivity were observed in tunica adventitia. Pattern of staining was altered slightly 7 days after SAH due to thickening of the vessel wall but other than being deposited around cells in areas of intimal proliferation of day 28 MCAs, there were no changes in type IV collagen immunoreactivity intensity or density in tunica media or adventitia after SAH (Table VI-3). Immunoreactivity for laminin exhibited a similar pattern to that of type IV collagen (Fig. VI-11, Table VI-3). Laminin immunoreactivity was observed within thickened tunica intima of day 28 MCAs.

Type V collagen immunoreactivity was observed in BAs and in MCAs at each time after SAH (Fig. VI-12). Fluorescence was most intense in tunica adventitia

although immunoreactivity was also observed in extracellular matrix of normal tunica media. Small amounts of immunoreactivity were present in normal tunica intima. No changes in type V collagen were observed until day 28 when hyperplastic areas of intima contained type V collagen deposited in interstitial areas (Table VI-3).

Histologic stain for elastic fibers showed numerous fine fibers within normal tunica media as well as occasional fibers in adventitia (Fig. VI-13). No increases in elastic tissue were seen after SAH. In sections of MCA 28 days after SAH, hematoidin pigment was present within tunica adventitia. Pigment was sometimes seen within macrophages but free pigment deposits were also observed.

Minimal immunoreactivity to fibronectin was observed in BA tunica media (Fig. VI-14). This increased slightly 7 days after SAH and even more prominently 14 days after SAH (Table VI-3). By day 28, immunoreactivity was decreasing. Tunica adventitia did not contain fibronectin immunoreactivity. Tunica intima of normal and SAH arteries exhibited minimal immunoreactivity to fibronectin which was increased in thickened intima 28 days after SAH. Immunoreactivity to fibrinogen was not present in normal basilar arteries and was only observed in small amounts in tunica media 7 days after SAH (Fig. VI-15, Table VI-3).

Staining for factor 8-related antigen revealed brown peroxidation products along endothelium of normal BA (Fig. VI-16). Areas of tunica intima lacking staining for factor 8-related antigen were not observed in MCAs at any time after SAH.

Cytoskeletal Proteins: In normal BA, immunoreactivity to α-actin and myosin was seen within smooth muscle cells in tunica media and in myointimal cells in tunica intima (Figs. VI-17 and VI-18). Both antibodies resulted in long, thin ellipse-shaped patterns inside cells, representing the normal, relaxed shape of these cells. On day 7, shorter, wavy patterns of staining were seen in both smooth muscle cells and in myointimal cells. Fluorescence in myointimal cells paralleled the convoluted shape of the internal elastic lamina. On day 14, pattern of staining was similar to BA with some shortening of smooth muscle cells still present. By day 28, a normal pattern was seen in smooth muscle cells in tunica media. Cells in excess intimal tissue observed on day 28 showed immunoreactivity to α-actin and myosin although even at day 28, significantly more α-actin positive intimal cells were not present compared to BA (Table VI-4). No changes in intensity or density of immunoreactivity to α-actin or myosin in tunica media or tunica adventitia were detected (Table VI-3).

Immunoreactivity to desmin was observed in smooth muscle cells in tunica media and in intima of BA (Fig. VI-19). No changes in intensity, density, or pattern of fluorescence were seen 7, 14, and 28 days after SAH apart from detection of desmin in areas of intima which showed proliferation by day 28 and changes in pattern of staining which would be expected to accompany obligatory vessel wall thickening due to arterial constriction on day 7 (Table VI-3). In BA, vimentin immunoreactivity was observed within smooth muscle cells, adventitial fibroblasts, and myointimal cells (Fig. VI-20). Other than increases due to deposition of vimentin in proliferated intima on day 28, no changes were seen in intensity or density of vimentin immunoreactivity over time periods examined (Table VI-3).

Measurement of Hydroxyproline:

A typical chromatogram showing results of amino acid content of a control BA is shown in Fig. VI-21. Content of hydroxyproline in MCAs from each time period is shown in Table VI-4. There were no significant changes when hydroxyproline was expressed per mol of leucine, per mg of dryed, defatted weight, or per mg of protein as estimated by amino acid analysis. There was no change in the degree of proline hydroxylation at any time after SAH (Table VI-4). Table VI-5 shows content of amino acids (expressed as residues per 1000) for arteries at each time after SAH. The only amino acid which appeared to change over time periods examined was γ-aminobutyric acid.

DISCUSSION

Extracellular Matrix Proteins

Extracellular matrix is a highly organized extracellular scaffold which cells produce *in vivo*.[28,31,32] It consists of collagens and elastins, noncollagenous glycoproteins, glycosaminoglycans, and proteoglycans and its synthesis is required for correct cell functioning during development, cell migration, and wound healing.[28,31] Arterial walls contain all these components, although collagen types in normal and diseased arterial walls have been most extensively studied.[1,30,31] There are few reports specific to cerebral arteries. Their collagen structure may differ from systemic muscular arteries of similar size due to the thinner adventitia and absence of external elastic lamina.[40] Of 11 genetically-distinct collagen types discovered thus far, types I, III, IV, V, VI, and VIII are present in blood vessels.[1,30,31] Types I and III are fibril-forming interstitial collagens which comprise 80% to 90% of total arterial collagen.[30] As observed in this study, they colocalize in extracellular matrix of tunica media, adventitia, and, in most studies, intima of normal arteries. Type IV collagen is a basement membrane constituent.[5] Basement membranes, which also contain laminin, heparan sulphate proteoglycan, and entactin, envelope smooth muscle cells, myointimal cells, and underlie endothelial cells.[5,16] Type V collagen is also present in normal arterial wall extracellular matrix.[30,31] It probably forms only small fibers and may help anchor the interstitial collagen matrix to basement membranes, although its structure and function *in vivo* are still poorly characterized. Type VI collagen has been detected by immunoflourescence in all layers of arterial wall although, like type VIII collagen, relatively few studies concerning its location and function have been reported.[30,31]

Collagens

Using semi-quantitative (immunofluorescence microscopy) and quantitative methods (amino acid analysis), we did not find increases in arterial collagens after SAH except after 28 days when intimal thickening developed. Apparently only small amounts of collagen were deposited in thickened intima since increased collagen could not be detected as increased hydroxyproline on amino acid analysis. Hydroxyproline is an amino acid found predominately in collagen.[36] Elastin is the only other major arterial wall protein which contains hydroxyproline, although it contains much less than collagen.[18,36] Lack of increase in hydroxyproline also suggests that elastin is not synthesized in cerebral arteries after SAH. Our results, using a histologic stain for elastin, also showed no changes after SAH.

Extent of proline hydroxylation influences secretion, stability, and degradation of collagen.[36] It is conceivable that short-lived increases in collagen occur after SAH, the increases resolving as VSP does. This might be reflected by lower levels of proline hydroxylation. We did not, however, detect changes in degree of proline hydroxylation at any time after SAH.

Few investigators have used quantitative methods to measure collagens in cerebral arteries after SAH. Nagasawa et al used colorometric assays for hydroxyproline to measure collagen content of dog BA following SAH and noted progressive increases which became significant by day 14.[33] They also reported substantial but insignificant increases in elastin.

Qualitative descriptions of light and electron microscopy of cerebral arteries after experimental SAH suggest there is increased collagen in tunica media of vasospastic arteries.[6,13,15,28,35,46] Some investigators failed to find fibrosis in arterial walls after SAH in humans and experimental animals.[8,12,15,20,37,42] Major changes in arterial wall configuration occur during vasoconstriction and previous interpretations of increased extracellular collagen during VSP may not have taken into account the marked widening of the intercellular space which results from vessel wall thickening during vasoconstriction.[47]

Arterial wall collagen deposition was suggested by several authors to cause artery stiffening noted on pharmacologic studies of vasospastic arteries.[2,3,48] Vasospasm

has been attributed to this loss of arterial compliance. Although late increases in collagen in tunica intima of arteries after SAH could account for stiffening of vessels, one would have to demonstrate reversal of these changes during resolution of VSP before attributing VSP to such changes.

Fibronectin, Fibrinogen, and Laminin

Fibronectin is a noncollagenous extracellular matrix glycoprotein with receptors for many proteins, including cell surface proteins and collagen.[28,32] It promotes cell adhesion and is characteristically expressed during development, cell migration, and in healing wounds where it is associated with myofibroblasts.[22,32,43] Increased fibronectin was found 7 days after SAH, although levels seemed to peak around day 14. Significant VSP was present 14 days after SAH and changes in fibronectin might contribute to maintenance of this narrowing based on functions of fibronectin within the fibronexus as a link between extracellular collagen and actin inside cells.[42] It is also conceivable that, based on these cross-linking functions of fibronectin, arterial wall stiffness is due to increased fibronectin or to a change in cross-linking of cells and collagen by fibronectin. Alternatively, fibronectin accumulates in areas of tissue injury [22,32,39] and therefore, increased arterial fibronectin after SAH also indicates medial smooth muscle has been injured. The increase follows the injury-producing period of intense vasoconstriction.

Fibrinogen immunoreactivity was detected transiently in tunica media 7 days after SAH and is consistent with previous reports and with literature documenting blood-arterial wall barrier breakdown after SAH.[19,41] Results of staining for factor 8-related antigen suggest major endothelial disruption is not required for leakage of fibrinogen into tunica media. Electron microscopy of vasospastic arteries may show loss of intercellular tight junctions which is presumably the basis for such leakage, rather than endothelial denudation.[15] Fibronectin is found in plasma and some the increase seen on day 7 could have been due to leakage across the disrupted blood brain barrier. By day 14, the barrier was apparently intact, and fibronectin deposition at this time was probably due to synthesis within arterial wall.

Laminin is a glycoprotein which is a major basement membrane component and is believed to bind cells, such as smooth muscle cells and endothelial cells, to their surrounding type IV collagen.[5,16] Differences in laminin in tunica media after SAH were not identified.

Role of Myofibroblasts

Smith et al used immunofluorescence techniques to detect type V collagen in a vasospastic cerebral artery 16 days after SAH.[46] This was taken as evidence for proliferation of myofibroblasts, which are derived from fibroblasts,[32,34] within tunica media and intima during VSP, as these cells are known to synthesize type V collagen. Our results show type V collagen is found in normal arteries. Therefore, the basis for invoking myofibroblasts in VSP is unclear. There are additional reasons to doubt that myofibroblasts are important in VSP. Smooth muscle cells also elaborate type V collagen [28,31] and thus, even if increases in type V collagen did occur during VSP, synthesis by smooth muscle could be responsible. Mammalian muscular arteries do not contain fibroblasts except in the tunica adventitia.[40] In the study of Smith et al type V collagen was noted in tunica media and tunica intima. This would require migration of fibroblasts through several layers of arterial wall, a unique phenomenon which has not been previously reported. Although our observations of α-actin within cells in proliferated intima at day 28 suggests these cells are derived from smooth muscle as most authors believe,[17] reports that myofibroblasts transiently express α-actin blurs the distinction between myofibroblasts and smooth muscle.[9,34,45] Myofibroblasts, however, do not generally synthesize basement membrane components, such as laminin.[10] While it cannot be shown that every cell in the wall was surrounded by laminin, laminin immunoreactivity within tunica media and proliferated intima on day 28 suggest it is not necessary to invoke any role for myofibroblasts in VSP. In addition, our finding

that increased myointimal cells are only present after VSP has resolved is consistent with previously published results [15,20] and negates any role for contraction of these cells in VSP since arteries containing them were relaxed.

Increases in fibronectin which we observed after SAH might be considered to support the myofibroblast theory since myofibroblasts secrete fibronectin in granulating wounds.[32,43] Vascular smooth muscle cells of synthetic phenotype secrete more glycoproteins than mature contractile cells and increased fibronectin is also consistent with change in smooth muscle phenotype within tunica media.[28]

Cytoskeletal Proteins:

Arterial wall collagen is deposited late after SAH, yet VSP often resolves within 2 weeks of SAH. Since arteries relax as small amounts of collagen are deposited within their intima, other processes may be fixing arteries in a narrowed state. These processes apparently are reversible. Kim et al found that in addition to stiffening of non-contractile structures, the contractile component of dog BA was altered to a state favoring arterial narrowing after SAH.[25] The basis for this change in smooth muscle function is unclear. Mayberg et al noted that VSP in piglets and rats was accompanied by decreased smooth muscle actin as judged by loss of immunoreactivity to α- and γ-actin isoforms.[29,35] Changes in characteristics of smooth muscle in response to culture *in vitro* and to injury have been reported. In general, mature smooth muscle cells *in vivo* are characterized by predominance of α-actin and smooth muscle isoforms of myosin, presence of desmin, ability to contract, and low rate of mitosis.[4,17] When cultured *in vitro*, smooth muscle cells alter expression of actin so that β-actin predominates and γ-actin is increased.[4,17] In addition, rough endoplasmic reticulum and golgi apparatus increase, desmin decreases, and cells may loose their ability to contract. This smooth muscle phenotype also secretes more collagen and noncollagenous glycoprotein than mature smooth muscle.[4,28]

Based on studies of smooth muscle phenotype after balloon injury to arteries, occurrence of intimal proliferation 28 days after SAH (and increase in fibronectin after 14 days) would suggest a transient decrease in tunica media α-actin occurred prior to migration of muscle cells into intima.[7,26] Changes were not detected in α-actin or desmin immunoreactivity within tunica media in this study. Duration of change in medial smooth muscle appears to be brief and arteries in this study may not have been examined during the time of changes. The transient nature and late occurrence of these events suggest that while they might somehow contribute to persistence of narrowing, they may also reflect response of the artery to injury incurred during vasospasm. We postulate that this injury is brought about by a prolonged, temporarily irreversible, nonphysiologic contraction of smooth muscle which underlies vasospasm.

Changes in smooth muscle myosin with injury have not been as extensively studied.[17] We found no differences in myosin immunoreactivity in tunica media after SAH.

While phasic smooth muscle contraction is mediated by actin-myosin interactions, the basis for tonic contraction is uncertain. Rasmussen and colleagues proposed that alterations, including phosphorylation, of the intermediate filament domain of smooth muscle, were responsible for force maintenance.[38] We did not detect changes in desmin or vimentin during VSP although this certainly does not exclude a role for alteration in configuration of these proteins, without increases in their concentration, which could temporarily fix the muscle cell in a contracted state.

Although the goal of amino acid analysis of cerebral arteries was to quantitate collagen, differences were not seen in hydroxyproline content. The only amino acid which did change was γ-aminobutyric acid. Pia-arachnoid arteries of several different species contain γ-aminobutyric acid and it is suspected that cerebral arteries are innervated by nerves containing this substance, although this has not been confirmed histologically.[21] It would be appropriate to examine the larger cerebral arteries to see if they do contain these nerves.

In summary, this study fails to find evidence that collagen is deposited in the arterial wall during VSP after SAH in monkeys. Intimal proliferation, which was noted only after arterial narrowing had resolved, contained small amounts of typical vessel wall collagens. Major changes in contractile and intermediate-filament proteins within smooth muscle cells were not seen during VSP. Intimal thickening and fibronectin deposition appear to be late responses of the arterial wall to injury incurred during VSP. These results are consistent with vasoconstriction being the most important mechanism causing lumen narrowing after SAH. It is also possible, however, that other unrecognized proteins or changes in degree of cross-linking between components of the extracellular matrix contribute to the persistant, resistant arterial narrowing which characterizes VSP. Further investigations into the mechanism by which subarachnoid blood clot causes prolonged smooth muscle contraction are required.

Table VI-1: Physiological Variables and Angiographic Vessel Diameters, by Group (Values are Means ± Standard Deviations).

	Day 7 Group		Day 14 Group			Day 28 Group		
Variable	Day 0	Day 7	Day 0	Day 7	Day 14	Day 0	Day 7	Day 28
weight (kg)	3.80±.65	3.66±.67	3.49±.55	3.05±.37	3.01±0.21	3.47±.79	3.23±.71	3.38±.88
MABP [a] (mmHg)	86±20	106±26	122±59	84±16	113±0	133±9	133±13	80±5
HR (min^{-1})	182±26	187±28	216±8	210±9	192±0	182±27	216±6	174±12
PO_2 (mmHg)	96±11	91±3	87±13	99±11	91±13	89±19	110±0	89±4
PCO_2 (mmHg)	45±14	39±3	42±9	37±5	40±0	41±2	40±1	40±1
Vessel Calibre								
C_3	1.42±.25	1.44±.23	1.91±.11	1.41±.18	1.42±.35	1.74±.23	1.32±.12	1.34±.27
C_4	1.04±.20	0.91±.19	1.01±.06	0.75±.20	0.92±.09	1.07±.13	0.84±.08	0.98±.09
MCA	0.84±.10	0.42±.15	0.90±.06	0.41±.06	0.76±.05	0.93±.08	0.43±.03	0.90±.13
ACA	0.70±.13	0.49±.14	0.80±.07	0.55±.25	0.66±.03	0.73±.06	0.65±.16	0.74±.05
VA	0.87±.16	0.84±.19	0.82±.19	0.88±.19	0.86±.16	0.79±.03	0.81±.11	0.75±.18
BA	0.99±.13	0.98±.29	1.20±.45	1.19±.40	1.18±.26	1.17±.38	1.04±.05	1.01±.26

[a] MABP, mean arterial blood pressure; HR, heart rate; C_3, extradural internal carotid artery; C_4 intradural internal carotid artery; MCA, sphenoidal segment of middle cerebral artery; ACA, precommunicating segment of anterior cerebral artery; BA, mid-basilar artery, VA, vertebral artery

Table VI-2: Per Cent Changes in Diameter for Each Measured Artery, by Group and Time After Subarachnoid Hemorrhage. Values are Means of Change Between the Specified Time Periods Obtained by Combining Measurements for Right and Left Sides ± Standard Deviations.

	Day 7 Group	Day 14 Group		Day 28 Group	
Vessel	Day 0-Day 7	Day 0-Day 7	Day 0-Day 14	Day 0-Day 7	Day 0-Day 28
C_3	5 ± 19	-26 ± 6 [c]	-26 ± 14	-22 ± 16 [c]	-23 ± 6 [a]
C_4	-10 ± 13	-25 ± 20	-8 ± 8	-21 ± 8 [c]	-7 ± 17
MCA	-50 ± 16 [b]	-55 ± 8 [b]	-16 ± 10 [a]	-54 ± 4 [a]	-3 ± 15
ACA	-27 ± 23 [b]	-30 ± 32	-17 ± 8 [a]	-10 ± 25	2 ± 9
BA	-1 ± 29	8 ± 15	6 ± 9	-6 ± 22	-13 ± 5
VA	-3 ± 10	0 ± 5	1 ± 16	2 ± 13	-4 ± 25

C_3, extradural internal carotid artery; C_4, intradural internal carotid artery; MCA, sphenoidal segment of middle cerebral artery; ACA, precommunicating segment of anterior cerebral artery; BA, mid-basilar artery; VA, intradural vertebral artery.

[a] $p < 0.05$, paired t-test

[b] $p < 0.001$, paired t-test

[c] $p < 0.005$, paired t-test

Table VI-3: Average Intensity of Immunofluorescence, Graded 0, +, or ++, in Adventitia and Media for Each Antigen and Time Period After Subarachnoid Hemorrhage

	Basilar Artery		Middle Cerebral Artery					
			Day 7		Day 14		Day 28	
Antigen	Adventitia	Media	Adventitia	Media	Adventitia	Media	Adventitia	Media
α-actin	0	++	0	++	0	++	0	++
myosin	0	+/++	0	+/++	0	+/++	0	+/++
desmin	0	+	0	+	0	+	0	+
vimentin	+	+	+	+	+	+	+	+
type-I collagen	++	+	++	+	++	+	++	+
type-III collagen	++	+	++	+	++	+	++	+
type-IV collagen	+	++	+	++	+	++	+	++
type-V collagen	++	+	++	+	++	+	++	+
fibronectin	0	+	0	+/++	0	++	0	+
fibrinogen	0	0	0	0/+	0	0	0	0
laminin	0	+	0	+	0	+	0	+

Table VI-4: Number of α-Actin Positive Cells per Millimeter of Arterial Circumference, Ratio of Moles Hydroxyproline (OHpro) to Moles Leucine, μmol OHpro per mg Dried Defatted Weight, Ratio of OHpro to Proline, and μmol OHpro per mg Protein, for Control Basilar Artery (BA) and Middle Cerebral Arteries at Each Time Period After Subarachnoid Hemorrhage (SAH). All values are Means ± Standard Deviations.

Days after SAH	No. of Samples	No. cells/mm	OHpro/Leucine	OHpro/mg weight	OHpro/mg protein	OHpro/proline
BA	4	31 ± 19	1.3 ± 0.1	0.32 ± 0.04	0.65 ± 0.03	0.75 ± 0.05
7	4	24 ± 11	1.2 ± 0.2	0.35 ± 0.13	0.58 ± 0.07	0.78 ± 0.07
14	2	28 ± 3	1.3 ± 0.1	0.30 ± 0.11	0.58 ± 0.07	0.80 ± 0.04
28	3	72 ± 42	1.3 ± 0.1	0.23 ± 0.06	0.70 ± 0.10	0.78 ± 0.04

Table VI-5: Amino Acid Content (Expressed as Number of Residues per 1000 ± Standard Deviation) of Normal Monkey Basilar Artery and of Middle Cerebral Artery at Each Time Period After Subarachnoid Hemorrhage.

	Basilar Artery	Middle Cerebral Artery		
Amino Acid	(n=4)	Day 7 (n=4)	Day 14 (n=2)	Day 28 (n=3)
Phosphoserine	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.1
Taurine	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
Phosphoethanolamine	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Aspartic acid	60 ± 5	60 ± 2	53 ± 3	60 ± 10
Hydroxyproline	64 ± 4	58 ± 6	63 ± 6	64 ± 9
Threonine	31 ± 3	31 ± 2	28 ± 1	28 ± 5
Serine	41 ± 3	42 ± 2	56 ± 17	40 ± 7
Glutamic acid	90 ± 6	90 ± 3	82 ± 5	93 ± 15
Proline	85 ± 7	75 ± 2	79 ± 3	84 ± 11
Glycine	262 ± 11	269 ± 10	277 ± 8	263 ± 12
Alanine	105 ± 3	107 ± 4	114 ± 4	114 ± 20
γ-Aminobutyric acid	1.4 ± 0.2	0.2 ± 0.1	0.7 ± 0	0.6 ± 0.8
Valine	50 ± 3	52 ± 6	53 ± 2	53 ± 10
Cystiene	3 ± 1	3 ± 1	3 ± 1	3 ± 1
Methionine	12 ± 1	12 ± 1	10 ± 1	8 ± 2
Isoleucine	24 ± 1	25 ± 3	17 ± 6	23 ± 4
Leucine	50 ± 3	51 ± 5	47 ± 1	51 ± 9
Tyrosine	15 ± 1	15 ± 2	13 ± 1	13 ± 2
Phenylalanine	23 ± 2	23 ± 2	21 ± 1	20 ± 4
Allohydroxylysine	5 ± 2	6 ± 1	5 ± 2	4 ± 2
Lysine	27 ± 2	27 ± 6	24 ± 5	29 ± 5
Histidine	10 ± 2	10 ± 2	8 ± 1	7 ± 2
Arginine	45 ± 1	47 ± 1	47 ± 1	46 ± 9

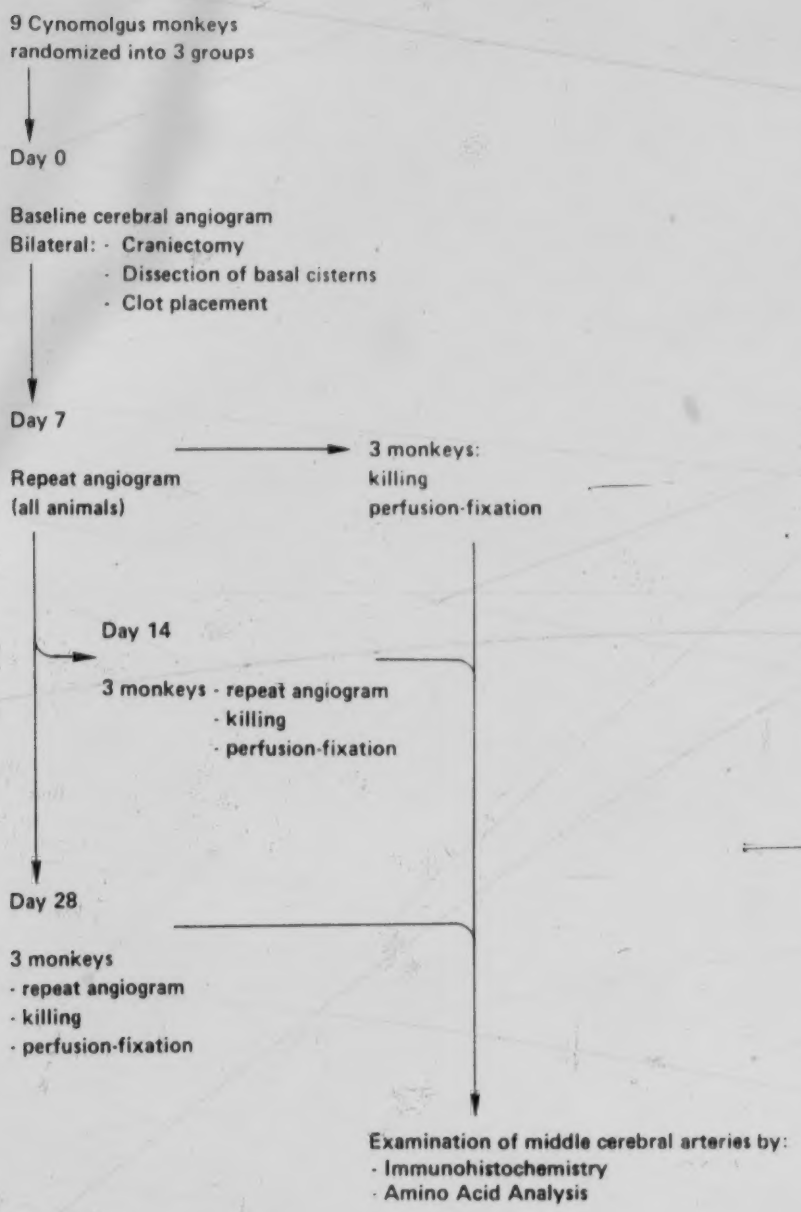

Figure VI-1: Diagram of experimental protocol for immunohistochemistry study and amino acid analysis of cerebral arteries at various time periods after subarachnoid hemorrhage.

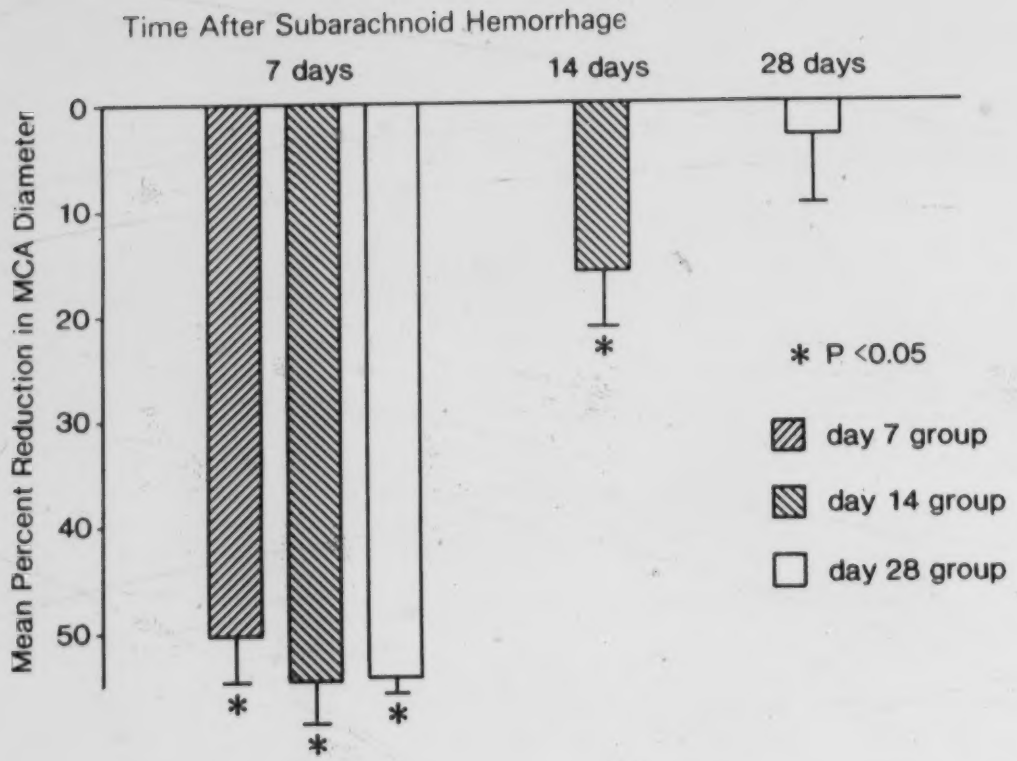

Figure VI-2: Mean per cent reduction in middle cerebral artery (MCA) diameter for each group at 7 days after subarachnoid hemorrhage (SAH) and at 14 and 28 days after SAH for monkeys killed at those times. Bars represent means ± standard error. Comparison of day 0 and day 7 angiograms within groups showed significant reduction in MCA diameter in each group ($p < 0.05$, paired t-test). Comparison of day 0 and day 14 angiograms also showed significant reduction in MCA diameter ($p < 0.05$, paired t-test).

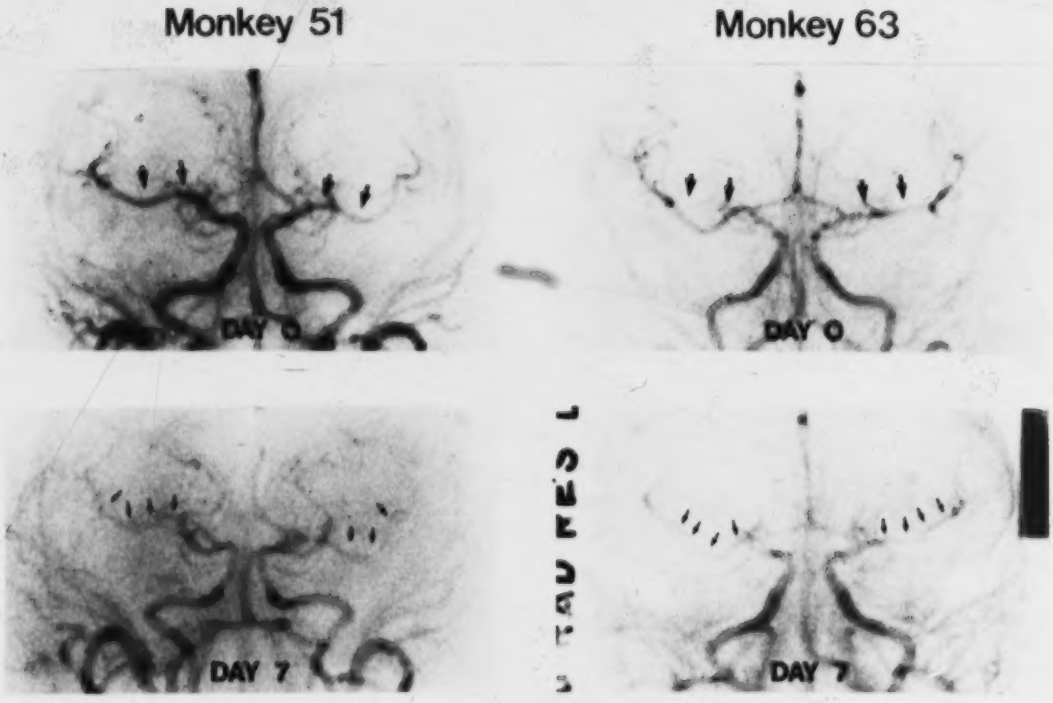

Figure VI-3: Representative angiograms taken at baseline (day 0) and on day 7 in monkeys killed 7 days after subarachnoid hemorrhage. There is significant narrowing of both middle cerebral arteries on day 7 (arrows).

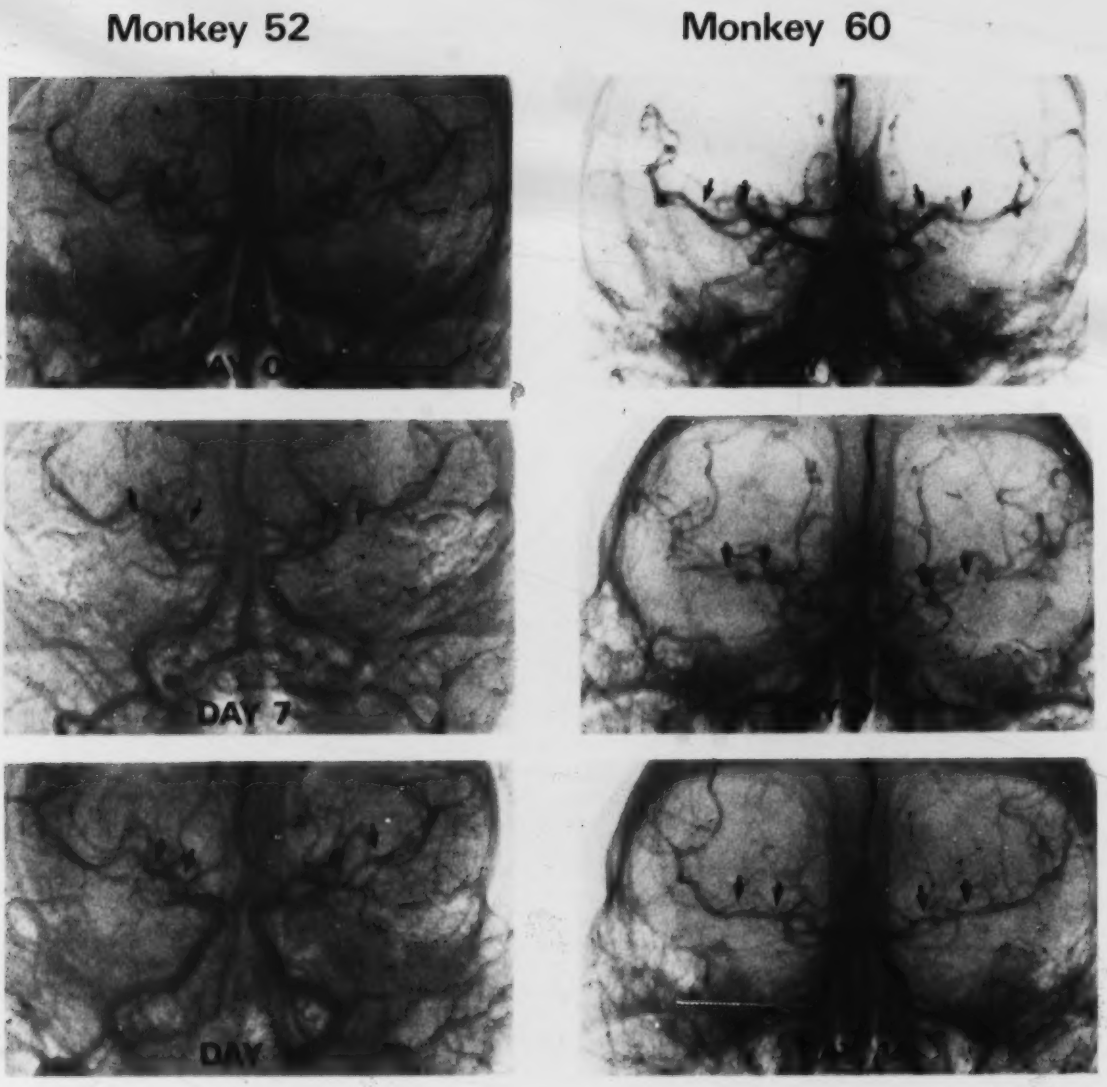

Figure VI-4: Representative angiograms taken at baseline (day 0) and 7 and 14 days after subarachnoid hemorrhage. In both animals there is significant narrowing of both middle cerebral arteries on day 7 which is resolving by day 14 (arrows).

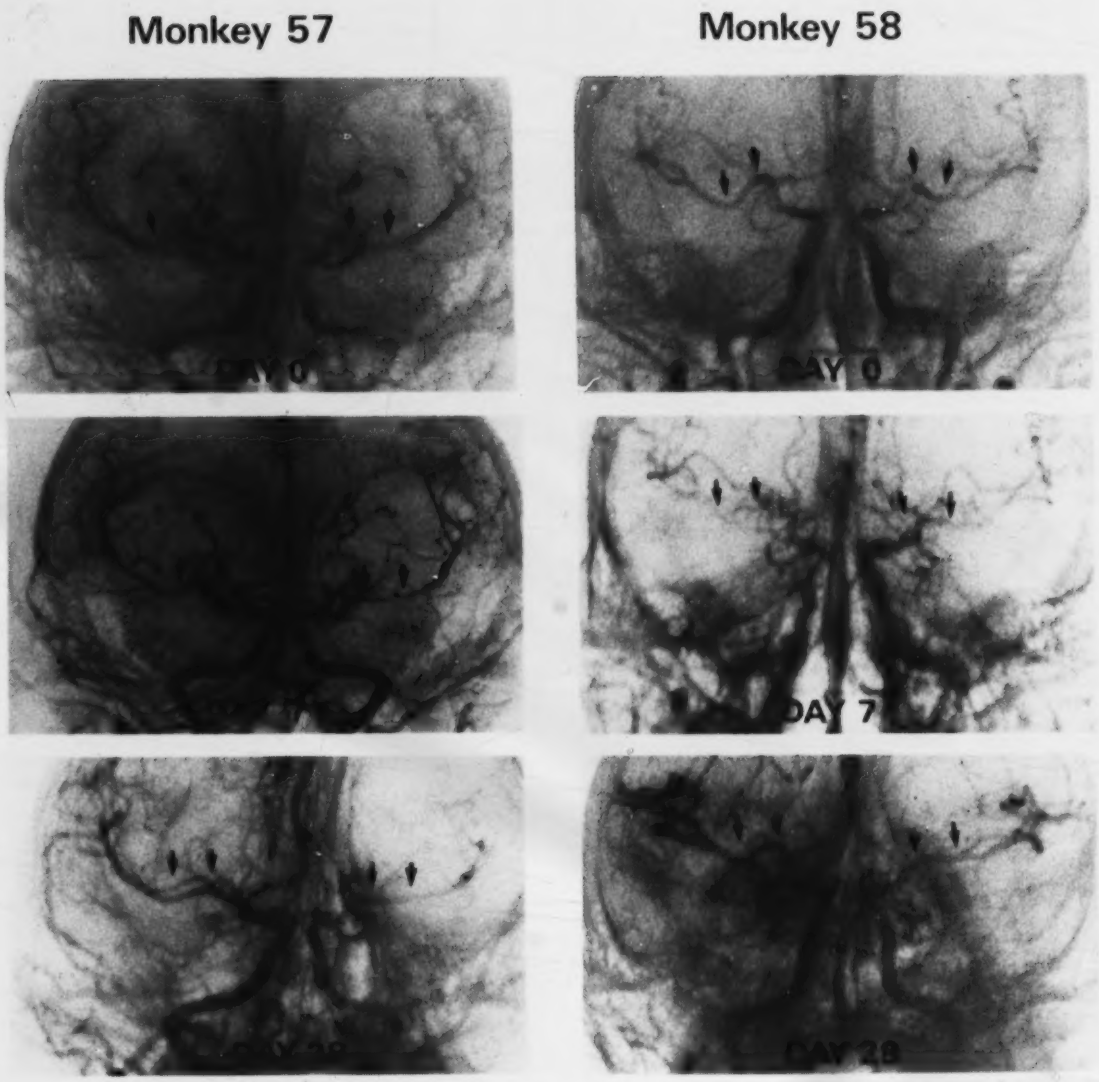

Figure VI-5: Representative angiograms taken at baseline (day 0) and 7 and 28 days after subarachnoid hemorrhage. In both animals there is significant narrowing of both middle cerebral arteries on day 7 which has resolved by day 28 (arrows).

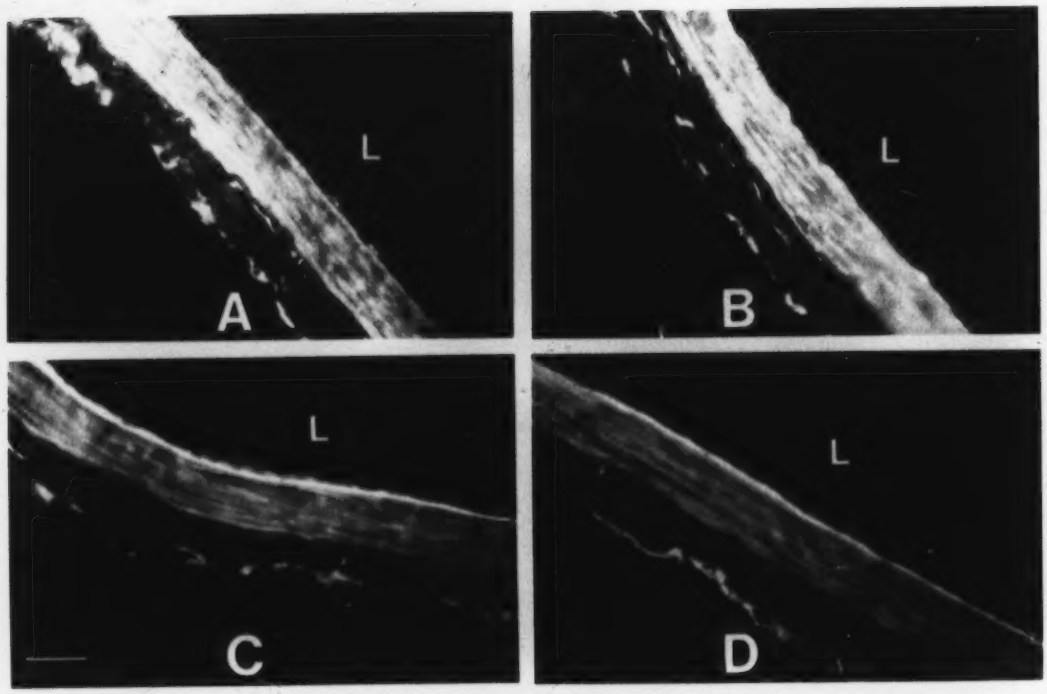

Figure VI-6: Fluorescence photomicrographs of normal basilar artery processed with primary antibody omitted and labelling by biotinylated secondary antibody and streptavidin-Texas red (A) or labelled with fluorescein isothiocyanate-conjugated secondary antibody (C). Only a low level of background tissue autofluorescence is observed under these conditions. Similarily, only minimal fluorescence is present when the primary antibody is replaced by normal goat immunoglobulin (B, biotinylated secondary antibody reacted with Texas-red streptavidin and D, fluorescein isothiocyanate-labelled secondary antibody). L, lumen. Scale bar in C (= 100 μm) applies to all 4 photomicrographs in the illustration.

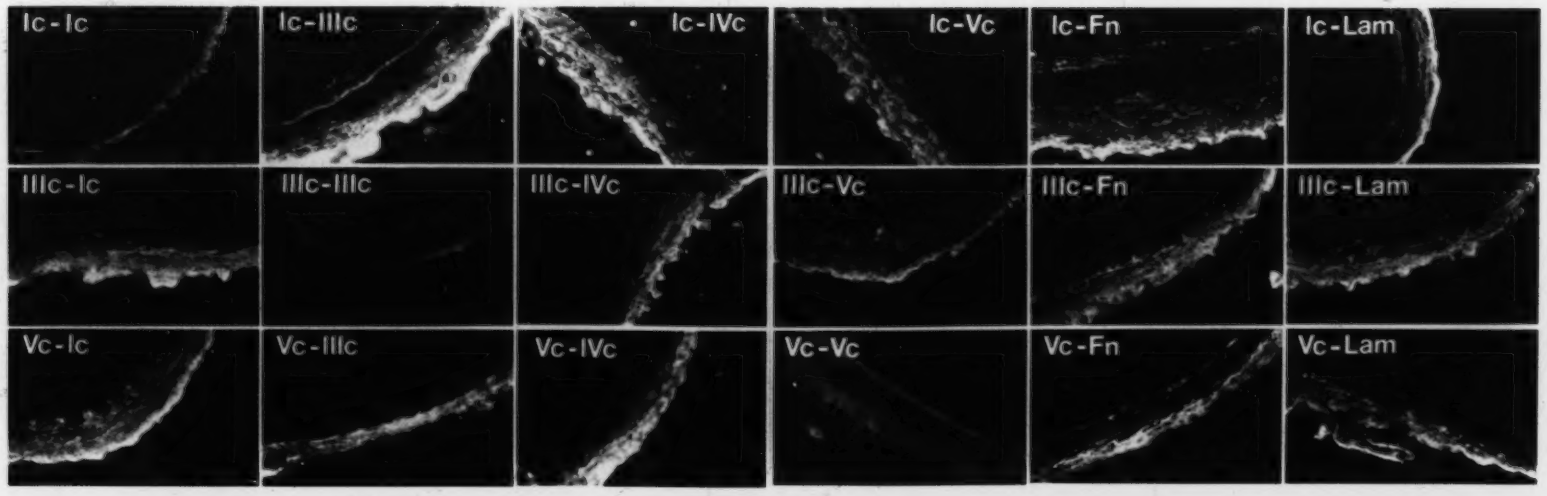

Figure VI-7: Fluorescence photomicrographs of absorption controls. The first symbol in the upper right of each photograph indicates the primary antibody used. The second symbol represents which antigen the primary antibody was incubated with prior to application to the section. Ic; type I collagen, IIIc; type III collagen, IVc; type IV collagen, Vc; type V collagen, Lm; laminin, Fn; fibronectin. Therefore, Ic-IIIc indicates type I collagen antibody preincubated with type III collagen. There is no change in staining after this procedure. For Ic-Ic, however, type I collagen antibodies bind type I collagen during preincubation and are not available to bind type I collagen in the arterial section, resulting in reduction in immunofluorescence. Scale bar (= 100 μm) in Ic-Ic applies to all photomicrographs in the illustration.

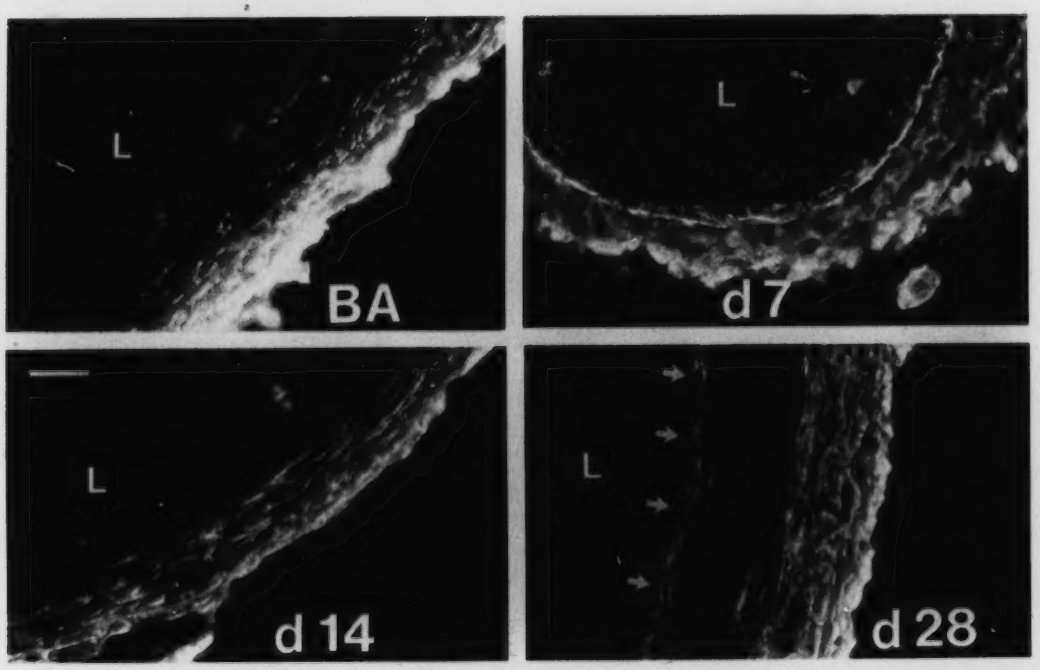

Figure VI-8: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-type I collagen antibodies. Significant changes in density or intensity of staining are not present apart from type I collagen immunoreactivity within thickened tunica intima on day 28 (arrows). L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

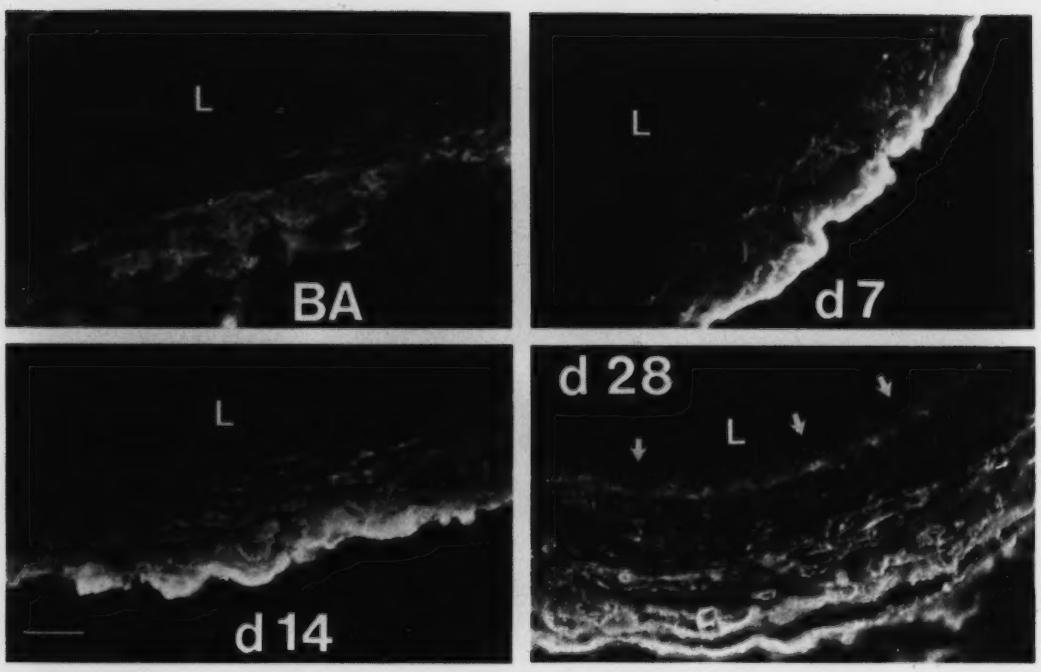

Figure VI-9: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-type III collagen antibodies. Significant changes in density or intensity of staining are not present apart from type III collagen immunoreactivity within thickened tunica intima on day 28 (arrows). L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

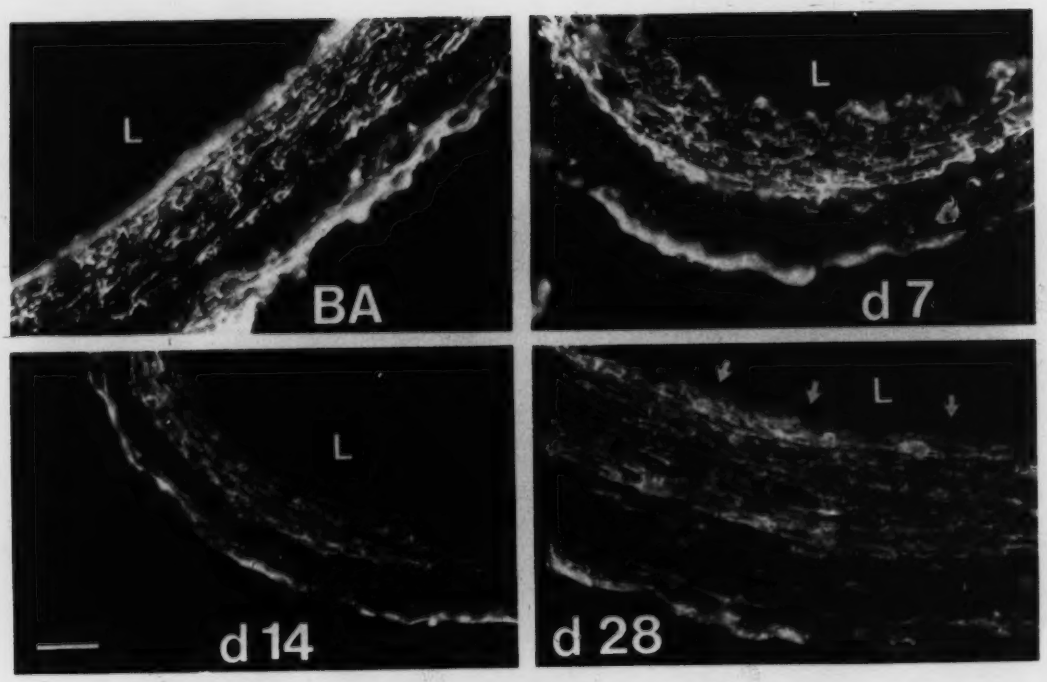

Figure VI-10: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-type IV collagen antibodies. Significant changes in density or intensity of staining are not present apart from type IV collagen immunoreactivity within thickened tunica intima on day 28 (arrows). L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

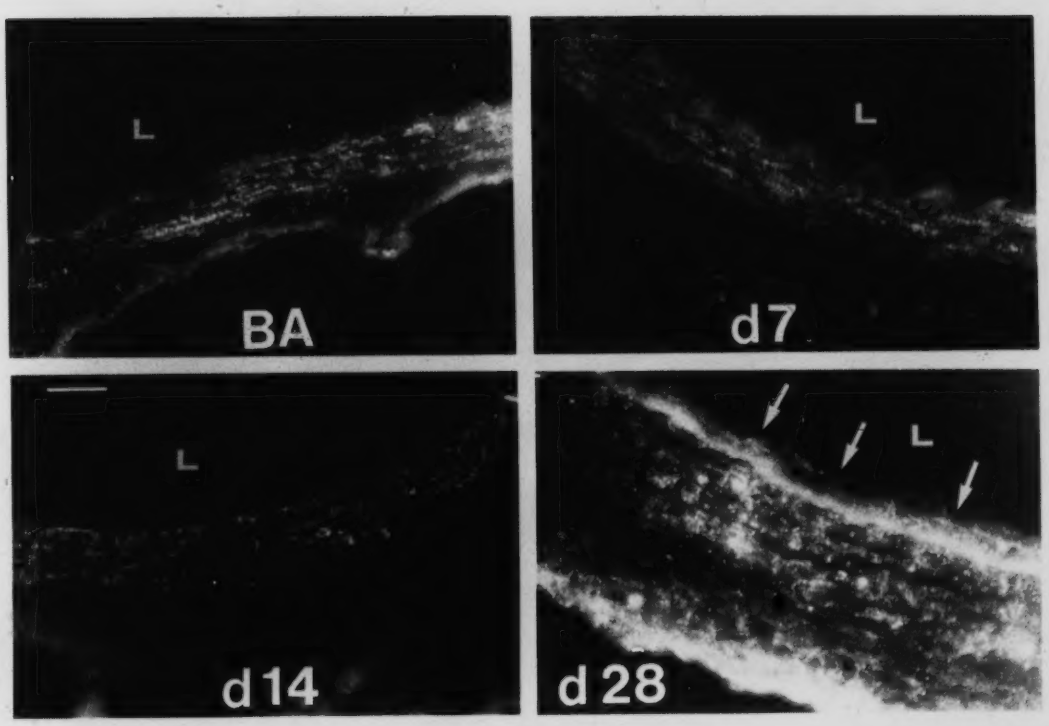

Figure VI-11: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-laminin antibodies. Significant changes in density or intensity of staining are not present apart from laminin immunoreactivity within thickened intima at day 28 (arrows). L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

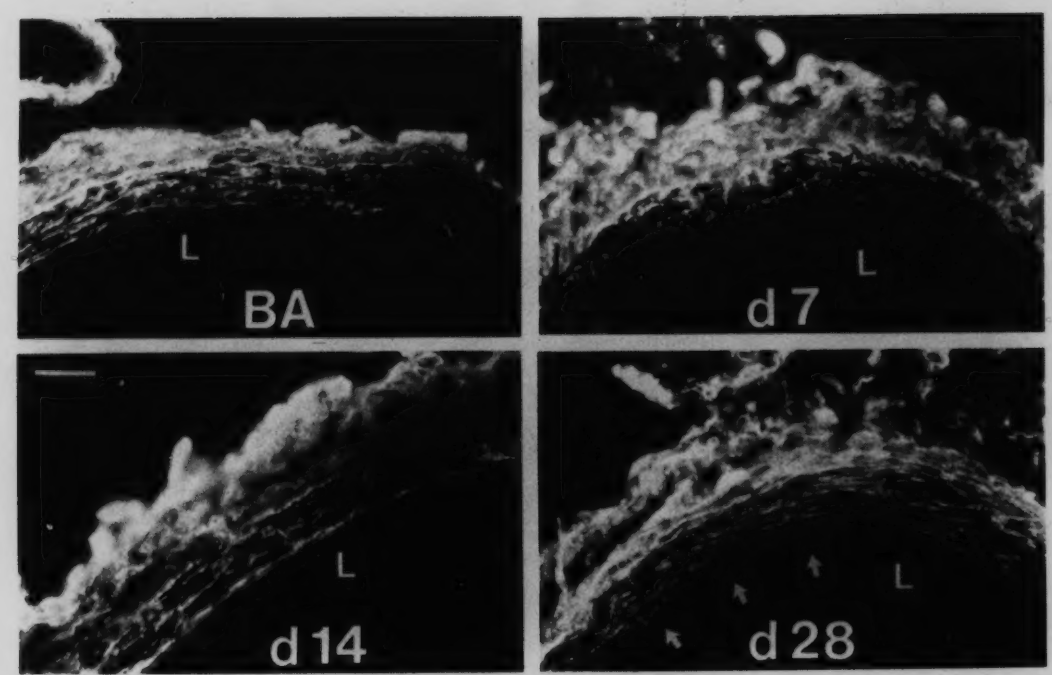

Figure VI-12: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-type V collagen antibodies. Significant changes in density or intensity of staining are not present apart from type V collagen immunoreactivity within thickened tunica intima on day 28 (arrows). L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

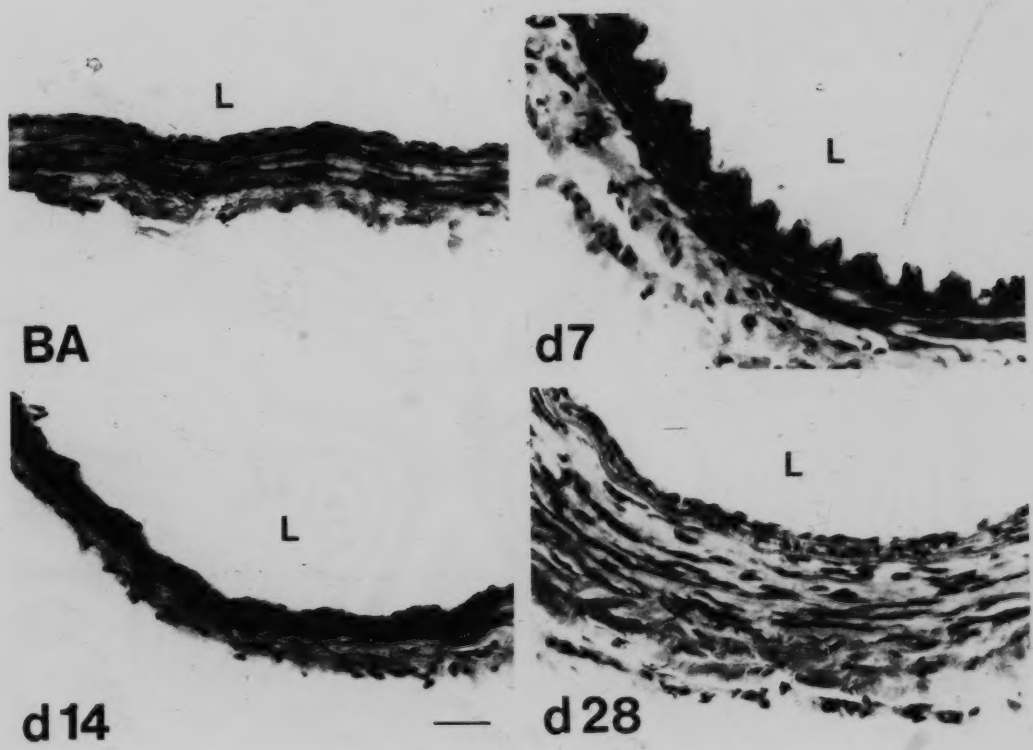

Figure VI-13: Photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and stained using Verhoeff's elastic stain. There are no increases in elastic tissue at any time after subarachnoid hemorrhage. Thickening of tunica intima is visible 28 days after subarachnoid hemorrhage. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

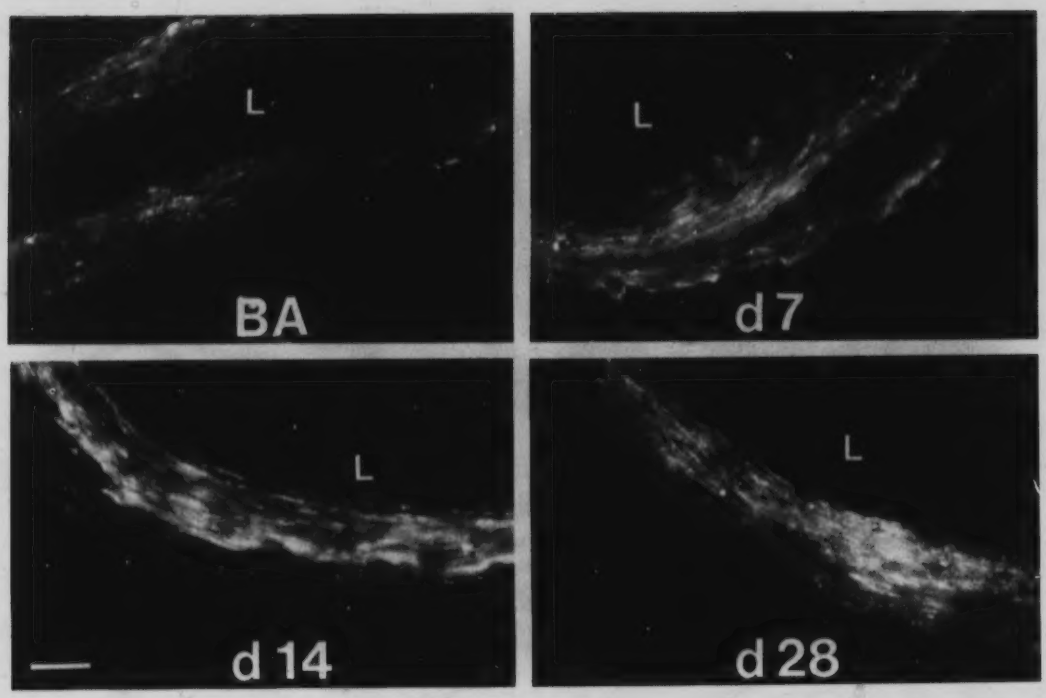

Figure VI-14: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-fibronectin antibodies. There is a slight increase in intensity of staining in tunica media on day 7. The increase is more obvious on day 14 and is subsiding 28 days after subarachnoid hemorrhage. L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

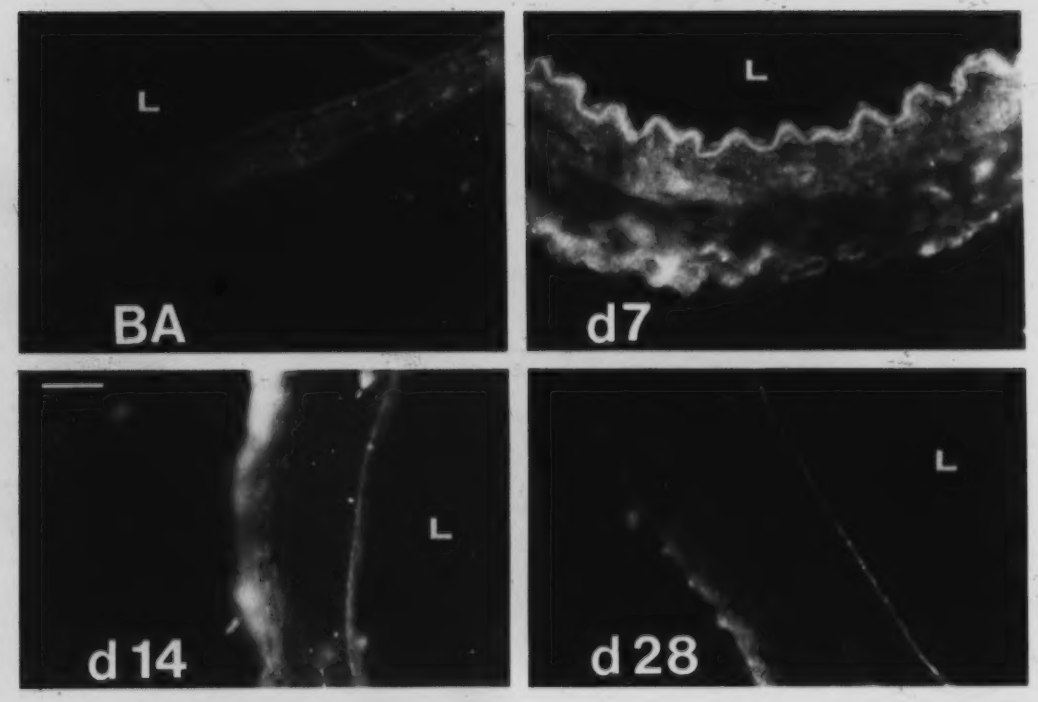

Figure VI-15: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-fibrinogen antibodies. There is a minimal staining in tunica media on day 7. At other time periods, immunoreactivity is not present. L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

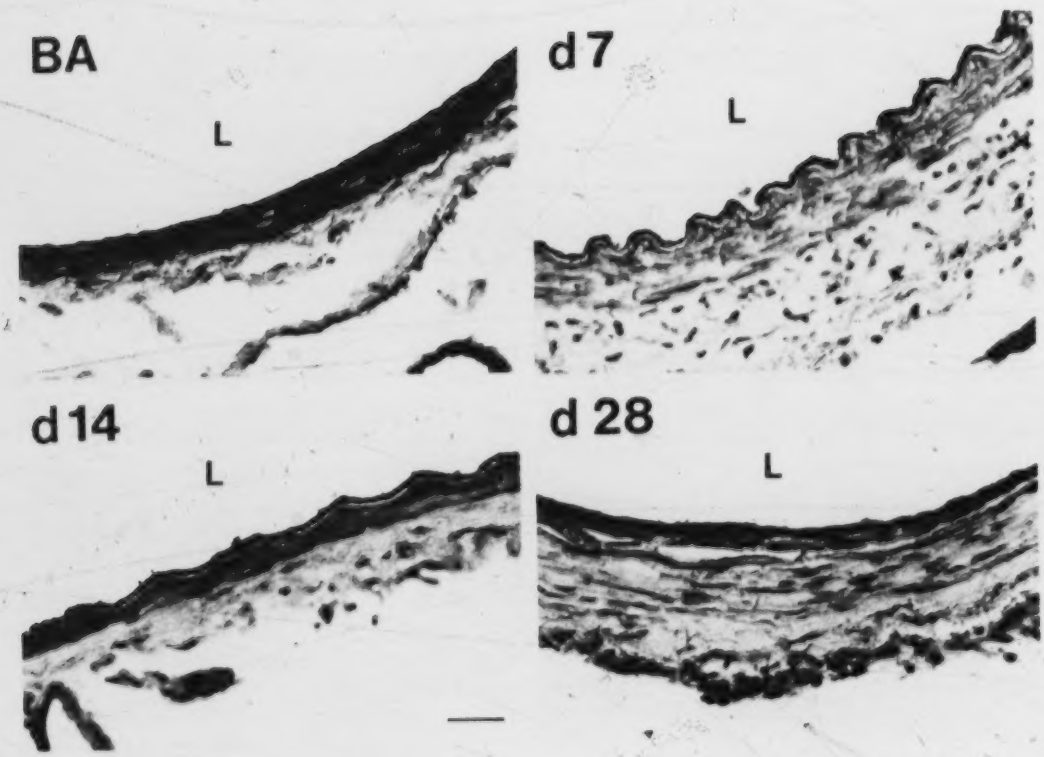

Figure VI-16: Photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and stained using immunoperoxidase technique with primary antibodies to factor 8-related antigen. There are no significant areas of loss of endothelium at any time after subarachnoid hemorrhage as judged by the continuous staining of endothelium at all times. L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

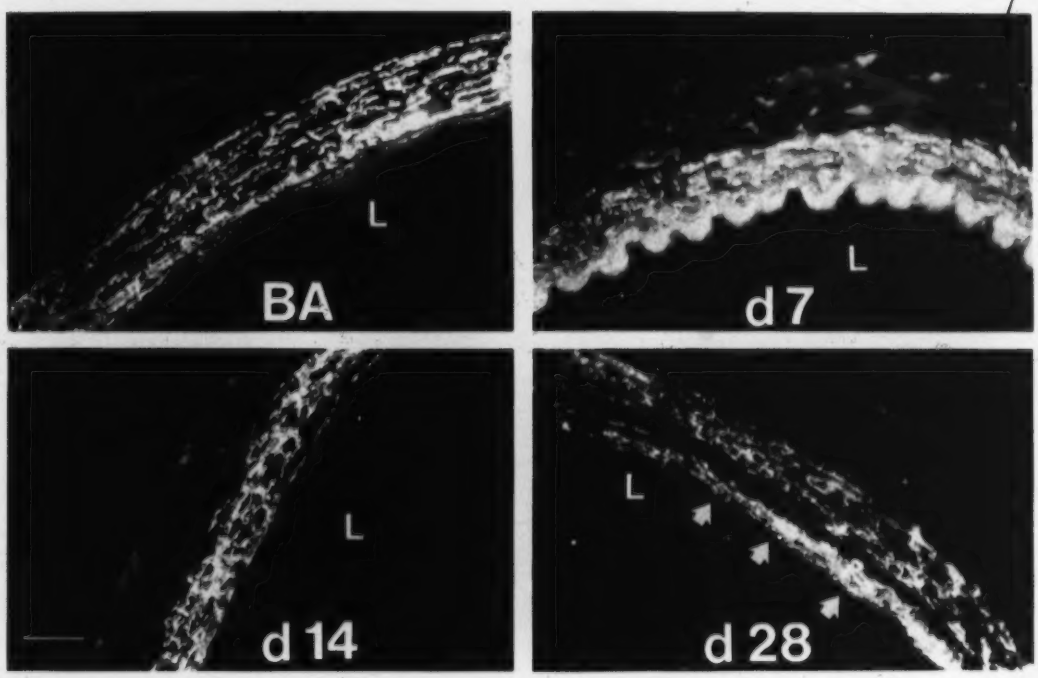

Figure VI-17: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-α-actin antibodies. Significant changes in density or intensity of staining are not present apart from increased numbers of cells containing α-actin immunoreactivity within thickened tunica intima on day 28 (arrows). L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

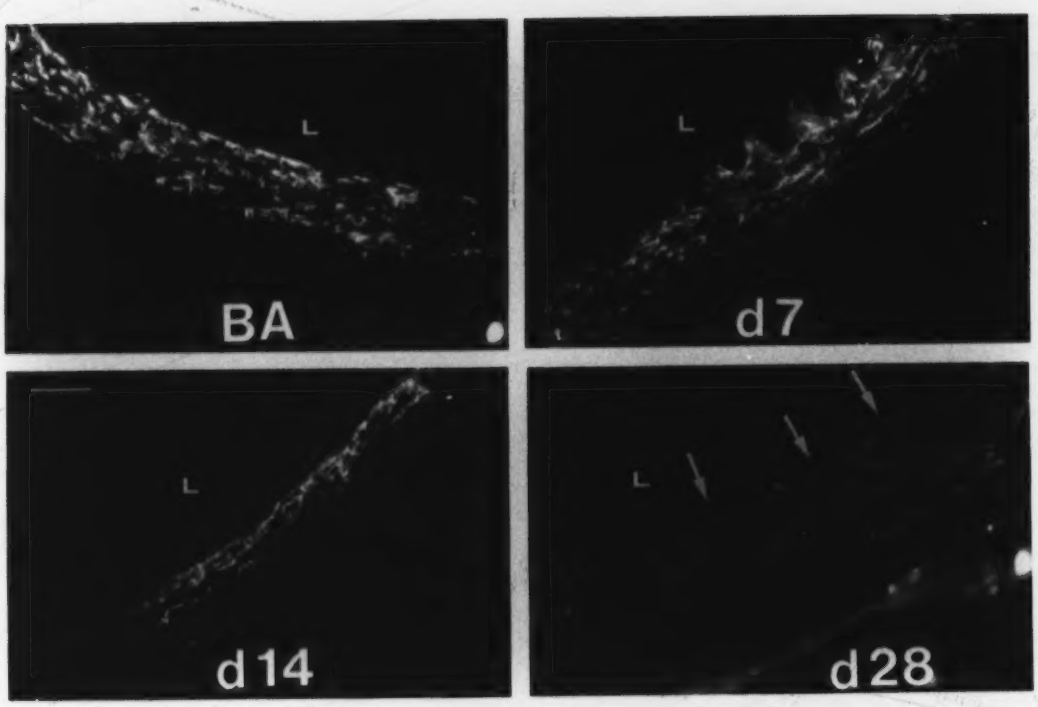

Figure VI-18: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-myosin antibodies. Significant changes in density or intensity of staining are not present apart from an increase in cells containing myosin immunoreactivity within thickened tunica intima on day 28 (arrows). L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

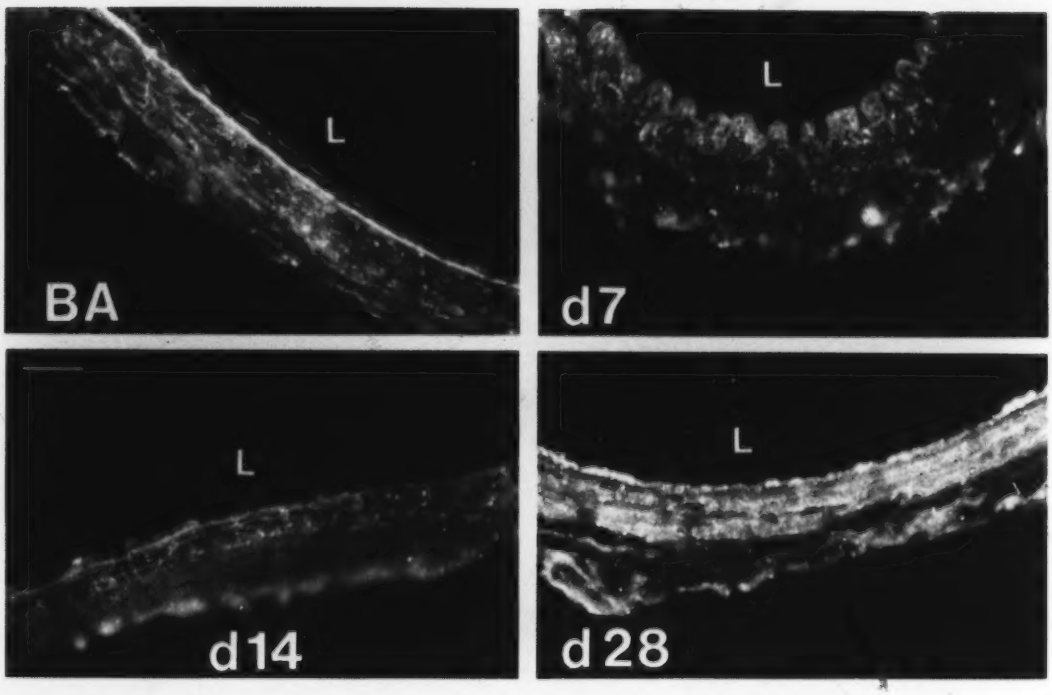

Figure VI-19: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-desmin antibodies. Significant changes in density or intensity of staining are not present. L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

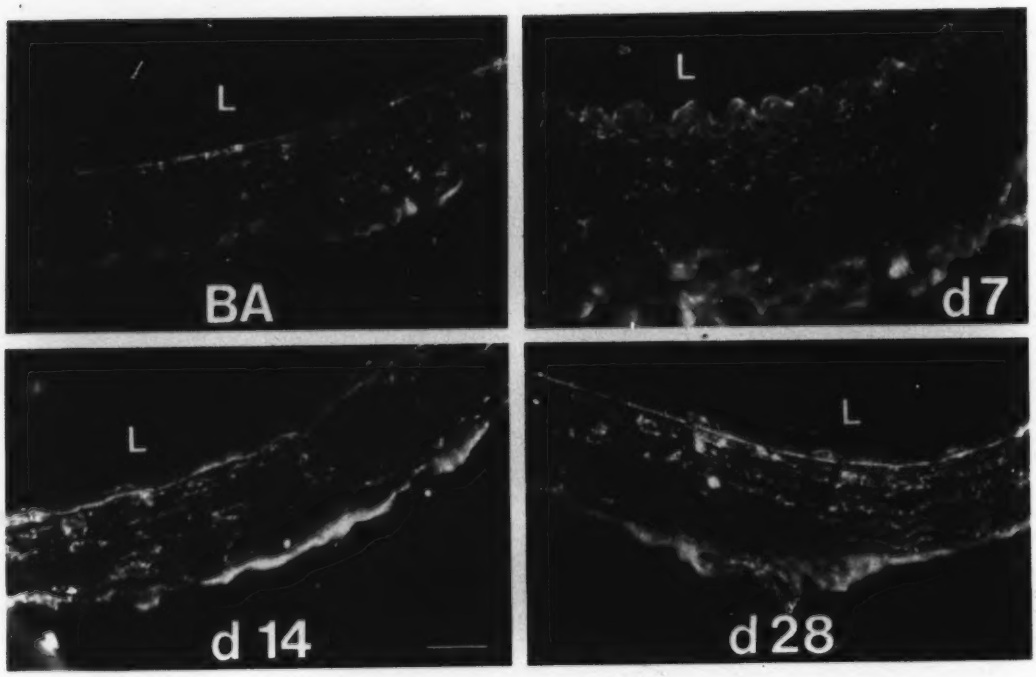

Figure VI-20: Fluorescence photomicrographs of normal basilar artery (BA) and of middle cerebral artery cross-sections from animals killed 7 (D7), 14 (D14), and 28 (D28) days after subarachnoid hemorrhage and exposed to anti-vimentin antibodies. Significant changes in density or intensity of staining are not present. L, lumen. Scale bar on day 14 photomicrograph (= 100 μm) applies to all 4 photomicrographs in the illustration.

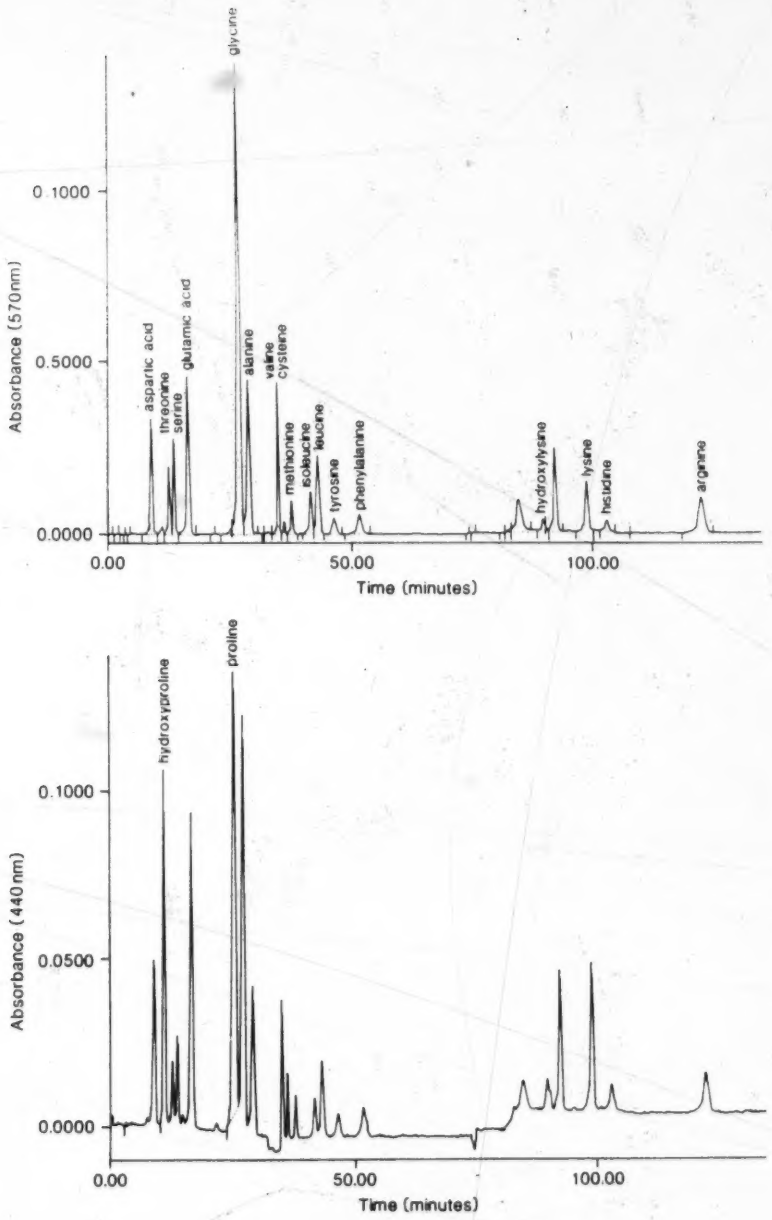

Figure VI-21: Representative chromatograms of amino acid analysis for a normal basilar artery. Upper chromatogram is based on detection at 570 nm whereas the lower chromatogram is detection at 440 nm. On the upper chromatogram, a small peak due to hydroxyproline can be seen eluting immediately after aspartic acid. Hydroxyproline peak is more prominent at 440 nm.

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CHAPTER SEVEN
SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

SUMMARY AND CONCLUSIONS

Oxyhemoglobin and Vasospasm

The first chapter of this work reviews which chemicals released from clotted subarachnoid blood might be important in pathogenesis of vasospasm (VSP) following subarachnoid hemorrhage (SAH). Evidence in favor of a role of hemoglobin includes its release into cerebrospinal fluid (CSF) after SAH during the time of VSP.[3] Erythrocytes are the component of blood necessary for VSP to develop in pigs,[32] cats,[8] and dogs.[35] The most prominent process occurring in the subarachnoid space during VSP is red blood cell hemolysis,[13] acute inflammation having peaked days before. Erythrocyte lysis is necessary for these cells to become vasoactive and the component released *in vitro* which is associated with contractile activity is oxyhemoglobin (OxyHb).[52] Despite this, few well conducted studies of the vasoactivity of OxyHb have been conducted *in vivo* and investigators continue to make statements such as "no single agent, however, has been proven to be responsible for either the constriction seen on angiography or the structural changes observed in the arterial wall".[53] Mechanisms by which OxyHb could cause VSP were reviewed. Oxyhemoglobin catalyzes lipid peroxidation,[1] damaging smooth muscle cells *in vitro*.[44] Lipid peroxides themselves are vasoactive.[41] Furthermore, recent evidence suggests superoxide anion radical or hydroxyl free radicals may mediate endothelium-dependent contractions in some blood vessels.[50] These free radicals could be produced by OxyHb in the subarachnoid space, contributing to smooth muscle contraction. Bilirubin, the major metabolite of heme, causes vasoconstriction.[9] Oxyhemoglobin inhibits endothelium-dependent relaxation,[24,29] augments release of endothelin from endothelial cells,[28] and increases arterial wall eicosanoid synthesis,[49] all of which could contribute to VSP. Finally, OxyHb damages perivascular nerves.[12,27] Which, if any, of these effects are important *in vivo* is unknown. Oxyhemoglobin has direct effects on vascular smooth muscle [44] but little is known about how it affects intracellular second messenger systems, calcium homeostasis, and the contractile apparatus.[51]

Experiments reported in Chapter Two demonstrated that OxyHb is capable of causing VSP in monkeys. The issue of vasoactivity of methemoglobin (MetHb) was resolved by using a pure solution of MetHb which was found to be inert. Contribution of metabolism of OxyHb to bilirubin in the genesis of VSP was found to be minimal as significant arterial narrowing did not develop, even after injection of massive doses of bilirubin. Based on these results, and previous evidence from the literature, it is suggested that OxyHb is the cause of cerebral VSP following SAH. Injection of supernatant fluid from autologous blood incubated *in vitro*, which contained the same total amount of hemoglobin, and the same concentration of OxyHb on day 6, resulted in the same degree of vasospasm as OxyHb itself. Analysis of CSF for malondialdehyde (MDA) showed an association between elevated MDA and VSP, although these results were not conclusive. It is still difficult to know if OxyHb causes VSP by directly damaging vascular smooth muscle, liberating lipid peroxides secondarily, or if OxyHb catalyzes breakdown of lipid elsewhere (for example, red cell membranes), and these lipid peroxides are the primary vasoactive agents. Lipid peroxides are produced during tissue damage and cerebral arteries which were examined by electron microscopy showed smooth muscle necrosis. Indeed, highest MDA levels were seen in groups with the worst VSP; those exposed to supernatant fluid and OxyHb. An intermediate level was seen in the bilirubin group, corresponding to mild arterial narrowing seen in these animals. Supernatant fluid contained MDA yet did not induce more severe VSP than pure OxyHb solution and this, in addition to the fact that OxyHb alone did cause VSP, could be taken as evidence that OxyHb exerts actions directly on the vessel wall. Reports that U74006F decreases lipid peroxidation in subarachnoid clot simply show the drug is effective at preventing peroxidation, whether

it occurs in blood clot or arterial wall.[21] Since it is effective, propagation reactions within arterial wall probably contribute to VSP.

Having demonstrated the ability of OxyHb to cause VSP, an artificial "clot" system was developed so VSP could be induced by simply placing a gel in the subarachnoid space which would slowly release OxyHb into CSF near the cerebral arteries. This approach eliminates the need for repeated intrathecal injections with their attendant difficulties, including risk of infection. Agarose-OxyHb gel was used to define the role of free radical mechanisms in genesis of OxyHb-induced VSP. In Chapter Three, ability of superoxide dismutase (SOD) and catalase to prevent OxyHb-induced VSP was examined. Limited monkey availability necessitated small numbers of animals in each group, hampering this study somewhat although valuable information was still obtained. It was confirmed that OxyHb, when combined with agarose, produced a gel resembling blood clot, which remained in the subarachnoid space and slowly released hemoglobin, producing VSP identical to that produced by blood clot. Drug delivery using osmotic pumps was easily accomplished with minimal intervention and low risk of infection. The dose schedule of SOD plus catalase chosen was ineffective in preventing VSP. Several reasons for this are possible. Oxyhemoglobin might not cause VSP through, or solely via, a mechanism mediated by free radicals. Alternatively, OxyHb may generate VSP through a free radical mediated reaction. Reports of dose-response curves of SOD [36] suggest that the particular doses and combination of enzymes utilized might not have been efficacious, while others may be. The enzymes used may be too large to penetrate into the vessel wall and provide protection against free radical reactions near smooth muscle itself. Finally, oxygen-derived free radical reactions, which could be prevented by SOD and catalase, may be critical only in the initiation of lipid peroxidation by OxyHb. Once initiated, peroxidation might involve radical species not susceptible to scavenging by SOD or catalase, such as peroxy or alkoxy radicals.

Mechanism of Vasospasm

Chapter One discusses significance of pathologic changes in cerebral arteries following SAH. One explanation forwarded is that VSP is smooth muscle contraction. Pathologic changes may be a result of damage to smooth muscle as a result of intense and prolonged contraction, presumably caused by OxyHb.[14] Although this theory proposes that muscle contraction is keeping the lumen narrowed throughout VSP, contraction may be aberrant in some way since vasospastic arteries from experimental animals have been shown not to relax or contract normally.[5,25,40] Vasospasm could be due to proliferation of cellular and extracellular elements in the arterial wall with lumen narrowing resulting from this mass of extra tissue encroaching on the lumen.[22] A third theory combines elements of the first two, hypothesizing that VSP starts out as smooth muscle contraction but that this soon resolves and pathologic changes in the artery, including fibrosis and inflammation, hold the vessel in a contracted state.[5] Clearly, the time course of pathologic changes is important to deciding which theory best explains VSP, although few studies have been done which follow VSP from its onset to resolution.

Chapters Four, Five, and Six describe studies which elucidate mechanisms that best characterize VSP following subarachnoid blood clot placement in monkeys. Computer-assisted image-analysis was used to measure arterial wall areas during VSP. Vasospasm due to whole blood did not cause increased total wall area. Intrathecal injections did increase wall area, but this was a minor and nonspecific effect most likely related to intrathecal catheter placement. The inflammation detected was not related to development of VSP. Magnitude of increases in total wall area were too small to be compatible with a role for proliferative vasculopathy or intimal proliferation during VSP. After injection of OxyHb, however, total wall area increased slightly and this could be consistent with arterial wall fibrosis and inflammation being important in maintaining lumen narrowing.

Study of morphology of smooth muscle cells of vasospastic cerebral arteries by scanning electron microscopy showed they had folded cell membranes, a feature associated with smooth muscle contraction. This confirms smooth muscle is contracted during VSP. However, similar folding could be produced by action of arterial wall collagen matrix on smooth muscle cells. Therefore, immunohistochemistry and amino acid analysis were used to define the time course of pathologic changes in arterial wall collagens, other extracellular matrix proteins, and cytoskeletal elements following SAH. There was no increase in collagen in vasospastic cerebral arteries until 28 days after SAH when intimal thickening was observed and contained immunoreactivity to typical vessel wall collagens. Fibronectin was deposited in arterial walls 14 days after SAH, during resolution of VSP. The time course of these changes indicate that arterial walls are injured after SAH, most likely during VSP.[7] The pathologic changes are the characteristic response of arterial wall to injury [7] and develop after VSP. Vasospasm seems best characterized, therefore, as severe vasoconstriction. This theory is consistent with experimental evidence indicating that VSP is, at least in some models, reversible with pharmacologic treatment.[34] Results of a randomized, controlled, double-blind study of the Ca^{2+}-channel antagonist nicardipine, which demonstrated efficacy of this drug against arterial narrowing following SAH, are also consistent with vasoconstriction and smooth muscle contraction being the processes which underlie VSP.[16,23] The mechanism of contraction remains unknown, although it probably involves processes not normally involved in smooth muscle contraction. There is, however, evidence to the contrary, including studies showing that vasospastic arteries are stiffer and less contractile than normal,[5,40] although at least some of the stiffening and loss of contractility found in vasospastic arteries is due to alteration in the contractile component of the arterial wall.[25] Unless one postulates that stiffening and loss of contractility are largely due to changes in smooth muscle which can reverse fairly rapidly once subarachnoid blood clot disappears, it is difficult to envision how VSP resolves, and why vasospastic arteries do not seem to restenose after transluminal angioplasty.[43]

The studies described in this thesis also do not rule out involvement in VSP of other extracellular components of the arterial wall, such as proteoglycans, or changes in cross-linking of known components, including collagen. It seems unlikely that the role of factors other than smooth muscle contraction will be adequately understood until biochemical mechanisms which underlie interactions of OxyHb with arterial wall are completely worked out.

RECOMMENDATIONS

Extracellular Mechanisms

These experiments leave some unanswered questions. Although the effect of OxyHb is not due to its metabolism to bilirubin, other mechanisms which might be involved include free-radical mediated processes,[1,44,50] endothelium-dependent effects,[24,28] release of vessel wall eicosanoids,[49] and neurogenic effects.[12,27] Most work on these processes has been done *in vitro* and few long-term *in vivo* studies have been performed which investigate the contributions to VSP made by free radicals, endothelium, eicosanoids, or cerebral innervation.

To investigate free radical mechanisms further, a dosage study of SOD plus catalase could be conducted as treatment for VSP induced by either whole blood or OxyHb-agarose gel. If a particular dose was effective in preventing VSP, this would support a role for superoxide anion radical, iron, and production of hydroxyl radical in pathogenesis of VSP. Failure of all doses could have several explanations. First, VSP might not be mediated by free radicals. Second, one could never be sure the enzymes actually got to smooth muscle, which is presumably where important free radical reactions occur. Third, free radicals other than those mentioned above could be involved. Although ferrous hemoproteins theoretically could act as Fenton reagents, this has never been proven [17] and it is possible that lipid peroxidation propagates in the

subarachnoid space using other active radicals, such as ferryl, perferryl, alkoxy, and peroxy radicals. Catalase and SOD do not scavenge these free radicals.

Most theories of free radical reactions and their involvement in VSP is based on study of lipid peroxidation. Problems with this approach are discussed above, and although one cannot measure free radicals directly, different techniques, such as microdialysis, could be employed to investigate this theory. Microdialysis tubes could be placed in the subarachnoid space next to vasospastic arteries after SAH.[4,54] This technique could be used to measure hydroxyl free radical production by detecting stable hydroxylation products of salicylic acid injected intravenously.[15] It might be possible to perfuse the dialysis fiber with conventional spin traps, such as 5,5-dimethylpyrroline-N-oxide (DMPO) or N-*tert*-butyl-α-phenylnitrone (PBN).[6,48] Microdialysis could be used to detect production of lipid breakdown products which could indicate processes occurring during VSP. Effects of various scavengers could be correlated with effects on free radical production in the subarachnoid space *in vivo*. Finally, one could assess the effects of intrathecal free-radical generation with, for example, xanthine, xanthine oxidase, and iron, on cerebral arteries *in vivo*.

Similar techniques could be used to investigate the role of eicosanoid release from arterial walls by OxyHb in genesis of VSP. Microdialysis and chromatography could detect prostaglandins, thromboxane, leukotrienes, and lipoxins in CSF adjacent to vasospastic cerebral arteries after SAH.[54] It would be useful to know concentrations of these agents, as well as concentrations of the various hydroperoxyeicosatetraenoic acids next to cerebral arteries during VSP since concentrations in lumbar CSF are too low to produce VSP.[2] Effects of inhibitors of eicosanoid synthesis could be tested against vasospasm induced by OxyHb gel and correlated with effects on degree of VSP.

To determine if endothelin is involved *in vivo* in VSP due to OxyHb, levels could be measured in CSF or in jugular venous outflow or endothelin immunoreactivity could be detected in vasospastic arteries using immunohistochemistry. Since endothelin is a protein, a complex method to estimate changes in endothelin production following SAH would be to use *in situ* hybridization to assess transcription and translation of endothelin genes. Endothelin is small enough to pass through dialysis membranes and one could conceivably set up an *in vivo* bioassay system where a dialysis fiber was placed adjacent to the middle cerebral artery. Seven days after SAH, perfusate from the fiber could flow onto, for example, a segment of artery devoid of endothelium, allowing one to assay endothelin and endothelium-derived relaxing factor. Alternatively, arteries could be excised and a bioassay set up *in vitro*. Finally, when inhibitors of endothelin receptors become available, it will be necessary to investigate effects of these drugs on VSP.

Although adrenergic nerves are probably not important in VSP, findings that cerebral arteries are innervated by a variety of peptide-containing nerves [10] which may release potent vasoactive neurotransmitters raises questions about their role in VSP. Changes in substance P-containing and vasoactive intestinal polypeptide-containing nerves do not correlate with VSP.[11,18] There is some evidence that calcitonin gene-related peptide and neuropeptide Y might be important in VSP although most of these investigations have employed a rat model of SAH.[11]

Intracellular Mechanisms

Findings in this thesis suggest the major process underlying VSP is smooth muscle contraction. Biochemistry of phasic smooth muscle contraction has been at least partially worked out.[20,38,39,47] Cell depolarization, stretch, or activation of cell surface receptors leads to increased cytosolic Ca^{2+}, either due to influx from the extracellular fluid or release from intracellular Ca^{2+} stores. Excess free Ca^{2+} binds calmodulin and this complex activates myosin light chain kinase, resulting in phosphorylation of the 20,000 dalton myosin light chain. This phosphorylation increases actin-activated Mg^{2+}-ATPase activity which leads to contraction. Events during sustained contraction seem to differ, since myosin light chain phosphorylation and intracellular Ca^{2+} decrease to basal or near-basal levels.[39,47] Tonic contraction may involve a latch-bridge state or a

involve a latch-bridge state or a second population of myosin-actin cross bridges which are regulated by a distinct Ca^{2+} system.[19,38] Another theory is that protein kinase C mediates tonic contraction by phosphorylating proteins in the intermediate filament domain of smooth muscle.[38] Contraction during VSP persists for days and is unusual in that contractile and relaxation responses of smooth muscle are impaired.[5,25,40] Smooth muscle is damaged and some cells are killed by sustained contraction.[14]

Some investigations have examined changes in signal transduction pathways, activities of various kinases and phosphatases, and phosphorylation patterns in vascular smooth muscle during contraction due to OxyHb or during VSP.[26,30,31,33,37,40,42,44-47,51] Oxyhemoglobin acutely increases inositol trisphosphate in cultured smooth muscle cells.[51] There is evidence that intracellular Ca^{2+} remains increased for up to 7 days when these cells are continuously exposed to OxyHb (Y. Takanashi, personal communication, April 1, 1991). With respect to VSP, activities of myosin light chain kinase and cyclic nucleotide-dependent protein kinases were not altered in dog basilar artery made vasospastic by 2 blood injections into the cisterna magna.[45] Most investigators report calmodulin antagonists have minimal or no effect on VSP in this model.[2,31,37] Kokubu et al demonstrated that an inhibitor of myosin light chain kinase, ML-9, significantly decreased arterial narrowing in a double-injection dog model,[26] although this beneficial effect also correlated with how much blood was around the basilar artery on day 7, suggesting other factors may have accounted for drug efficacy. Antagonists of cell surface membrane voltage-gated Ca^{2+} channels are also known to exert little dilatory action on VSP. The newer intracellular Ca^{2+} antagonists, such as HA1004 and HA1077, may diminish VSP, at least in dog models of VSP.[42,46] Finally, Matsui and colleagues reported that VSP in a 2 hemorrhage dog model was reversed by protein kinase C inhibitors, H7 and staurosporine.[2,30,31,33] Analysis of basilar artery segments in VSP showed elevated levels of diacyl glycerol and increased phosphatidylcholine turnover.[31] Although H7, staurosporine, and HA1077 are not entirely specific in their reported actions, these results suggest a role for protein kinase C in the contraction associated with VSP.

It would be useful to perform a series of experiments to investigate effects of OxyHb *in vitro* and of subarachnoid clot *in vivo* on the various mediators of phasic and tonic contraction. Using cultured vascular smooth muscle cells and arterial segments *in vitro* one could assess effects of OxyHb at various times on intracellular Ca^{2+}, inositol polyphosphates, diacyl glycerol, activities and locations of protein kinase C, myosin light chain kinase, and cyclic nucleotide-dependent protein kinases, and phosphorylation of myosin light chain, caldesmon, and the other intermediate filament proteins. How these factors change during onset, progression, and resolution of VSP induced *in vivo* by blood clot or OxyHb gel could be assessed. Some work is currently underway although there has been minimal work on phosphorylation patterns in vasospastic smooth muscle.

Management of VSP by early operation, securing of the bleeding aneurysm, prophylactic nimodipine administration, and hypervolemic, hypertensive, hemodilution therapy have reduced morbidity and mortality due to VSP after SAH.[13] Despite these advances, a simple, efficacious, and safe treatment for VSP still does not exist. Hopefully, with a better understanding of the pathogenesis and mechanisms of VSP, effective treatments will be discovered.

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